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# Rapid Genetic Identification and Mapping of Enzymatically Amplified Ribosomal DNA from Several *Cryptococcus* Species

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**Detailed restriction analyses of many samples often require substantial amounts of time and effort for DNA extraction, restriction digests, Southern blotting, and hybridization. We describe a novel approach that uses the polymerase chain reaction (PCR) for rapid simplified restriction typing and mapping of DNA from many different isolates. DNA fragments up to 2 kilobase pairs in length were efficiently amplified from crude DNA samples of several pathogenic *Cryptococcus* species, including *C. neoformans*, *C. albidus*, *C. laurentii*, and *C. uniguttulatus*. Digestion and electrophoresis of the PCR products by using frequent-cutting restriction enzymes produced complex restriction phenotypes (fingerprints) that were often unique for each strain or species. We used the PCR to amplify and analyze restriction pattern variation within three major portions of the ribosomal DNA (rDNA) repeats from these fungi. Detailed mapping of many restriction sites within the rDNA locus was determined by fingerprint analysis of progressively larger PCR fragments sharing a common primer site at one end. As judged by PCR fingerprints, the rDNA of 19 *C. neoformans* isolates showed no variation for four restriction enzymes that we surveyed. Other *Cryptococcus* spp. showed varying levels of restriction pattern variation within their rDNAs and were shown to be genetically distinct from *C. neoformans*. The PCR primers used in this study have also been successfully applied for amplification of rDNAs from other pathogenic and nonpathogenic fungi, including *Candida* spp., and ought to have wide applicability for clinical detection and other studies.**

Clinical identification of pathogenic fungi is often hampered by a number of significant problems. Morphological and cultural characters of many fungi are often simple and may show considerable phenotypic plasticity. Most zoopathogenic fungi also do not produce a sexual stage (teleomorph) and instead are identified only from their asexual reproductive stage (anamorph). Classification of fungi based solely on anamorphic features is often inherently artificial, however, and may not therefore reflect true phylogenetic relationships. For these reasons, molecular approaches should be especially useful for genetic identification of fungi and for establishing their taxonomic relationships.

The genus *Cryptococcus* is a heterogeneous group of nonfermentative, encapsulated yeast species, which includes several important human pathogens. Among these, *Cryptococcus neoformans* has been increasingly reported from immunocompromised patients, particularly those infected with human immunodeficiency virus. Although the perfect state (teleomorph) of *C. neoformans* has been identified in the basidiomycete genus *Filobasidiella*, most isolates of *C. neoformans* and other *Cryptococcus* spp. rarely, if ever, produce sexual stages under laboratory conditions (3, 8, 17).

One recent approach for establishing genetic relationships in medically important fungi is by analysis of restriction fragment length polymorphisms (RFLPs) in their DNAs. For example, different serotypes of *Histoplasma capsulatum* may be distinguished by their RFLPs in both mitochondrial DNA and ribosomal DNA (rDNA) (21). Magee et al. (13) also reported that restriction site and length variation within the rDNA locus from several *Candida* spp. corresponded well with previous recognized taxonomic groupings and that intraspecific variation for restriction phenotypes might even be useful for strain identification.

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Several limiting features of conventional restriction analyses using Southern blot hybridization for routine identification and systematics are a requirement for relatively clean DNA samples as well as substantial time and resources necessary for preparing DNA hybridization membranes and probes. Sensitivity of Southern blots is also sometimes affected by poor signal/noise ratios caused by poor hybridization of heterologous probes or high background signals on blotting membranes. To circumvent some of these potential problems, we have used an approach that allows direct analysis of a desired target sequence without the necessity of blotting or hybridization. Our approach utilizes the polymerase chain reaction (PCR) to enzymatically amplify target sequences in vitro by a factor of over $10^8$, starting from minute amounts of genomic DNA template (19, 21). The level of sensitivity, specificity, and adaptability of the PCR has resulted in many applications, including efficient cloning, rapid direct sequencing, and highly sensitive detection (2, 12, 19, 22). The basic PCR protocol involves the use of two oligonucleotide primers (which flank a target DNA sequence) to initiate DNA synthesis on opposing strands of a target sequence, using a heat-stable DNA polymerase. Repeated cycles (20 to 30) of denaturation, primer annealing, and DNA synthesis result in a theoretical doubling of target sequence during each cycle. Automation of the PCR by using a thermal cycler device allows simultaneous amplification of DNA from many samples, which can then be used directly for analysis.

In this study, we examined DNA sequence variation in the rDNA of several *Cryptococcus* spp. by analysis of enzymatically amplified rDNA fragments. The various rRNAs and their respective DNA coding regions are known for their value as evolutionary markers, since they contain regions of both high and low sequence variability (5). The rDNA repeat of *C. neoformans* is organized similarly to that of most other fungi and contains coding regions for 17S, 5.8S, and 25S

TABLE 1. *Cryptococcus* and *Candida* strains used, along with data on their sources and teleomorphs (if known)

| Taxon and strain | Source |
|---|---|
| Taxa associated with a *Filobasidiella* teleomorph | |
| *C. neoformans* 132 | Type culture, serotype D, =ATCC 32719, IFO 0608, CBS 132 |
| *C. neoformans* 101 | J. Perfect, serotype A |
| *C. neoformans* C3D | J. Perfect, serotype A, clinical isolate |
| *C. neoformans* 3501 | J. Perfect, serotype D, mating type α, =ATCC 34873 |
| *C. neoformans* 3502 | J. Perfect, serotype D, mating type **a**, = ATCC 34874 |
| *C. neoformans* n32 | J. Perfect, serotype B |
| *C. neoformans* n34 | J. Perfect, serotype C |
| *C. neoformans* n33 | J. Perfect, serotype C |
| *C. neoformans* 602 | J. Perfect, serotype A, nonencapsulated strain |
| *C. neoformans* D321 | W. Schell, clinical isolate |
| *C. neoformans* n10 | J. Perfect, clinical isolate from AIDS[a] patient |
| *C. neoformans* n11 | J. Perfect, clinical isolate from AIDS patient |
| *C. neoformans* n12 | J. Perfect, clinical isolate from AIDS patient |
| *C. neoformans* n31 | J. Perfect, clinical isolate from AIDS patient |
| *C. neoformans* n16 | J. Perfect, Busse-Bueske isolate made in 1893 |
| *C. neoformans* n18 | J. Perfect, clinical isolate, serotype A or D |
| *C. neoformans* n25 | J. Perfect, clinical isolate |
| *C. neoformans* n27 | J. Perfect, clinical isolate, serotype A or D |
| *C. neoformans* n35 | J. Perfect, serotype B or C |
| Taxa associated with a *Filobasidium* teleomorph | |
| *C. albidus* var. *aerius* 155 | Type culture, =ATCC 10665, NRRL Y-1399, CBS 155 |
| *C. albidus* var. *albidus* 142 | Type culture, =ATCC 10666, CBS 142 |
| *C. albidus* var. *diffluens* 160 | Type culture, =ATCC 12307, IFO 0612, CBS 160, NRRL Y-15 |
| *C. albidus* var. *ovalis* K6-7 | Type culture, =ATCC 22460, S. Goto K6-7 |
| *C. albidus* D322 | W. Schell, clinical isolate |
| *C. uniguttulatus* 103.87 | W. Schell, clinical isolate |
| *C. uniguttulatus* 162.86 | W. Schell, clinical isolate |
| Taxa not associated with a teleomorph (asexual) | |
| *C. ater* 4685 | Type culture, =ATCC 14247, CBS 4685, NRRL Y-66 |
| *C. laurentii* 139 | Type culture, =ATCC 18803, CBS 139, IFO 0609, 0906 |
| *C. laurentii* D318 | W. Schell, clinical isolate |
| *C. laurentii* 108.87 | W. Schell, clinical isolate |
| *C. laurentii* 142.89 | W. Schell, clinical isolate |
| *Candida albicans* 666 | J. Perfect |
| *Candida albicans* C9 | J. Perfect |

[a] AIDS, Acquired immunodeficiency syndrome.

RNAs along with their respective spacer segments (18). In this study, we demonstrate the utility of restriction analysis using PCR products for (i) amplification and restriction mapping of rDNA from *Cryptococcus* species, (ii) identification of species- and strain-specific RFLPs, (iii) linking of anamorphic and teleomorphic life stages, and (iv) phylogenetic analysis.

## MATERIALS AND METHODS

**DNA preparation.** The fungal isolates used are listed in Table 1. DNA from 14 isolates of *C. neoformans* and *Candida albicans* was prepared from protoplasted cells (16) provided by John R. Perfect (Department of Medicine, Duke University). The remaining isolates were grown overnight in 25 ml of potato dextrose broth with shaking. Because the PCR does not necessarily require high-quality DNA for successful amplification, we adopted a "minimal prep" procedure for simplified cell lysis and DNA extraction (11). Yeast cells were harvested by centrifugation (5,000 × *g*, 10 min, 5°C), washed once with cold distilled water, recentrifuged, and suspended in 3 to 5 ml of extraction buffer (0.15 M NaCl, 50 mM Tris hydrochloride, 10 mM disodium EDTA, 2% sodium dodecyl sulfate [pH 8.0]). The suspended cells were then incubated at 65°C for 1 h, extracted one time with phenol-chloroform-isoamyl alcohol (25:24:1 by volume), and precipitated with 0.6 volume of isopropyl alcohol. Additional isopropanol (up to 1 volume) was added to lysates that did not produce a visible precipitate after 5 min at room temperature. The DNA precipitates were collected by centrifugation (10,000 × *g*, 15 min, 5°C), washed in ice-cold 80% ethanol for 5 min, and dried under vacuum. The final DNA pellet was suspended in 500 μl of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA) and stored at −20°C until use. Concentrations of DNA samples were estimated by running small samples on agarose minigels against genomic DNA standards.

**Handling procedures and precautions for using PCR.** Because of the risk of cross-contamination from handling of many identical PCR reactions, it was necessary to follow strict precautions as outlined previously (6). Many of these precautions simply require good sterile technique and awareness of potential contamination sources. Dedicated pipettors, electrophoresis chambers, storage space, and reagents for handling PCR products were assigned before the experiments were performed. Negative controls (omitting DNA template) were also included in each PCR experiment.

**Enzymatic amplification of DNA with use of the PCR.** DNA samples were diluted before the PCR was performed. A sample of each miniprep DNA (10 to 15 μl) was electrophoresed into low-melting-point agarose gels (0.6% SeaPlaque agarose; FMC Bioproducts), using Tris-borate-EDTA buffer as described previously (14). After staining

TABLE 2. Oligonucleotide primer sequences used to amplify fungal rRNA

| Name | Nucleotide sequence, 5'→3' | Position on *S. cerevisiae* rRNA |
|---|---|---|
| SR1-R | TACCTGGTTGATTCTGC | 1–17, 17S RNA |
| 5.8S | CGCTGCGTTCTTCATCG | 51–34, 5.8S RNA |
| 5.8S-R | TCGATGAAGAACGCAGC | 34–51, 5.8S RNA |
| LR1 | GGTTGGTTTCTTTTCCT | 73–56, 25S RNA |
| LR3 | GGTCCGTGTTTCAAGAC | 654–638, 25S RNA |
| LR5 | ATCCTGAGGGAAACTTC | 968–952, 25S RNA |
| LR6 | CGCCAGTTCTGCTTACC | 1141–1125, 25S RNA |
| LR7 | TACTACCACCAAGATCT | 1448–1422, 24S RNA |
| LR7-R | AGATCTTGGTGGTAGTA | 1422–1448, 25S RNA |
| LR12 | GACTTAGAGGCGTTCAG | 3126–3110, 25S RNA |

with ethidium bromide, the high-molecular-weight bands corresponding to genomic DNA (100 to 200 ng of DNA) were cut out from the gels, diluted into 1 ml of sterile distilled water, and dissolved by heating at 65°C for 10 min before preparation of the PCR reactions. Oligonucleotide primers were prepared by using standard phosphoramidite blocking and deblocking chemistries on an Applied Biosystems 380B synthesizer. The oligonucleotide primers used and their locations on the rDNA are shown in Table 2. The PCR reactions were set up with Amplitaq DNA polymerase (U.S. Biochemicals) in either 100- or 25-μl volumes, using buffer conditions recommended by the manufacturer. Thirty PCR cycles were performed on an automated thermocycler device (Perkin-Elmer-Cetus), using the following parameters: 94°C denaturation step (1 min), 50°C annealing step (45 s), 50 to 72°C ramp (1 min), and primer extension at 72°C (1 min). A final 7-min incubation step at 72°C was added after the final cycle to ensure complete polymerization of any remaining PCR products. PCR products were checked by running 1 to 2 μl of each reaction mixture on agarose minigels.

**Restriction analysis of PCR products.** After removal of

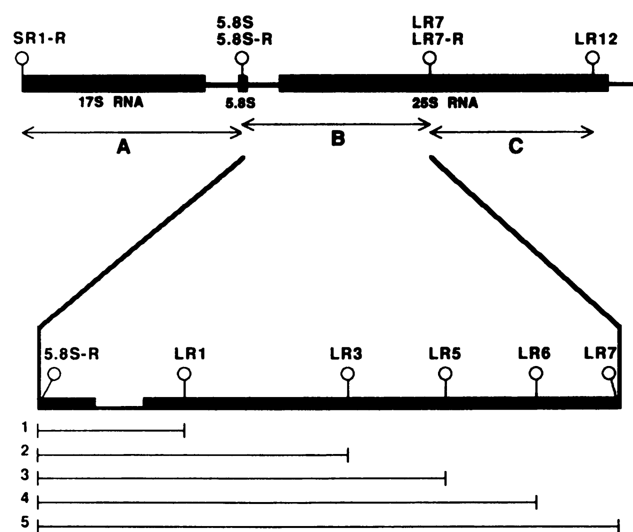


FIG. 1. Generic map of a portion of the rDNA repeat showing the locations of oligonucleotide primer sites (given in Table 1) used to amplify rDNAs from *Cryptococcus* spp. and other fungi. The expanded region of fragment B shown at the bottom also indicates the locations of additional primer sites used to determine the order of restriction sites by PCR mapping.

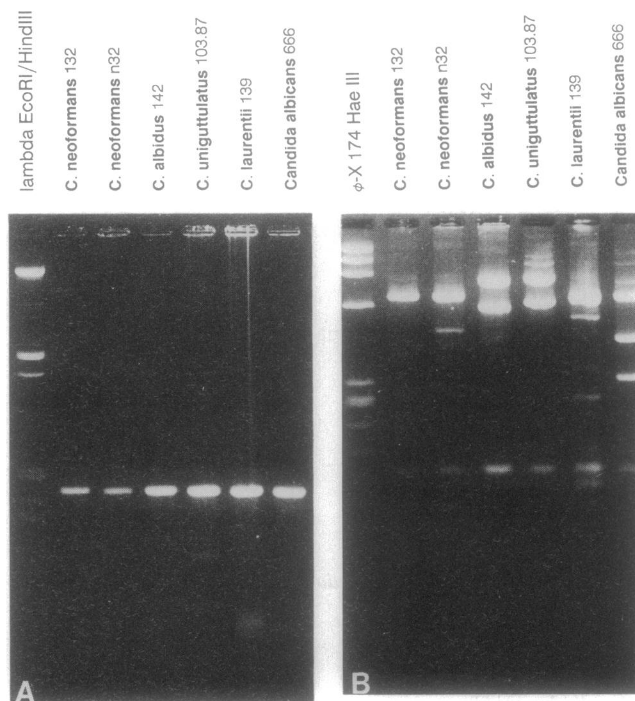


FIG. 2. PCR fingerprint analysis of rDNAs from six strains of *Cryptococcus* and *Candida* spp. (A) Oligonucleotide primers LR7-R and LR12 were used to amplify fragment C from Fig. 1 (corresponding to the 3' half of the 25S RNA cistron); 5 μl of each PCR reaction product was electrophoretically separated in 0.8% gels. (B) Enzymatically amplified C fragments from the six strains were digested with *Hha*I and separated on 3% agarose. Restriction analysis reveals species- or strain-specific patterns among the six strains.

some of the mineral oil overlay from the PCR reactions, the amplified products were extracted once with 1 volume of phenol-chloroform-isoamyl alcohol. The aqueous portion was removed, and DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.0), followed by 3 volumes of 95% ethanol. Ample time was given for precipitation (>1 h at −20°C). The precipitates were collected by centrifugation for 15 min in a microfuge, washed in 80% ethanol, dried under vacuum, and resuspended in 50 μl of TE. For restriction analysis, 5 to 10 μl of each PCR product was digested in a 20-μl volume, using *Taq*I, *Ava*II, *Hin*fI, *Hha*I, or *Msp*I under conditions supplied by the manufacturer (Promega Biotec). The restriction reactions were separated by electrophoresis in 3% agarose gels (2.25% NuSieve agarose, 1.75% Seakem GTG agarose; FMC Bioproducts), using φX174 *Hae*III-digested fragments as molecular weight standards. After staining, the gels were photographed over a UV transilluminator to record the results.

**Restriction analysis of rDNA by PCR mapping.** To map restriction sites within the rDNA fragments, we used a novel application of the PCR which greatly facilitates restriction mapping by obviating the need for Southern blotting and probe hybridization. This method, which we call PCR mapping, is based on restriction analysis of incrementally larger PCR fragments sharing a common endpoint (Fig. 1). The positions of different restriction fragments (and their terminal restriction sites) from the largest PCR product are determined from their serial appearance in digests of successively larger fragments. The B fragments from several *Cryp-*

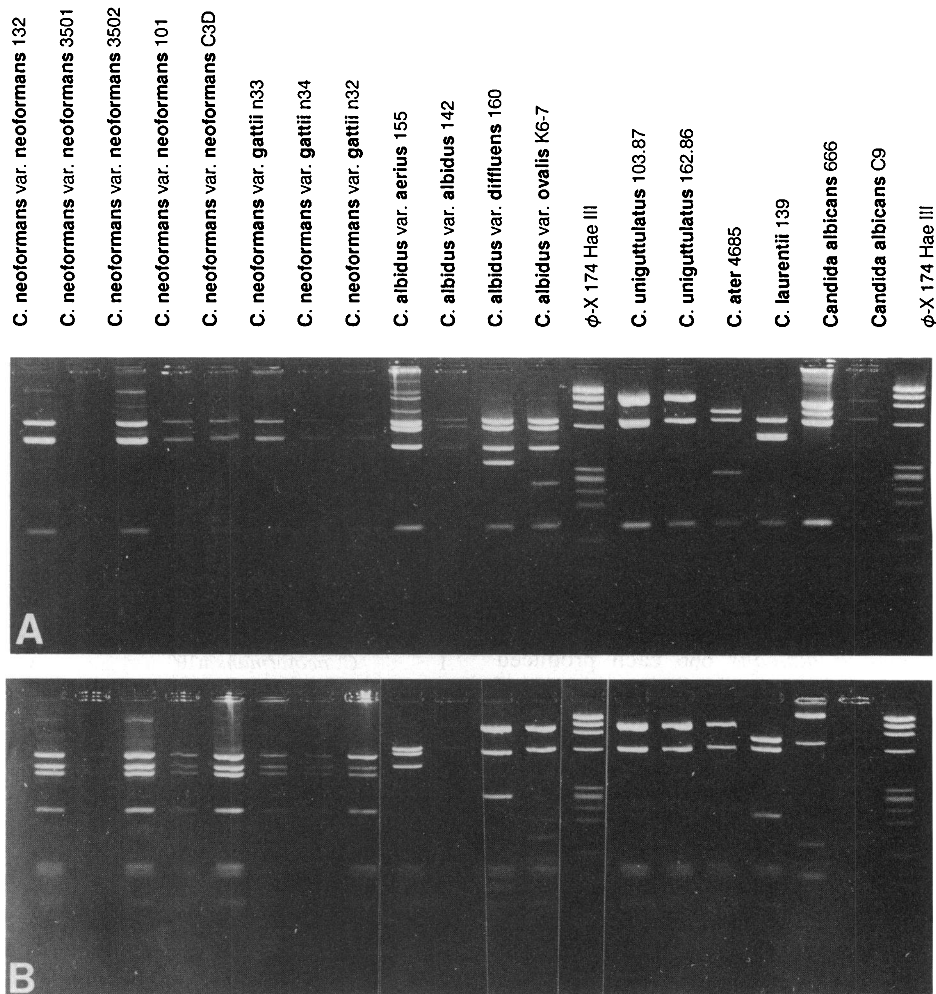


FIG. 3. PCR fingerprint analysis of rDNA (fragment B) from 18 reference strains of *Cryptococcus* and *Candida* spp. (A) PCR fingerprints produced after digestion with *Ava*II; (B) PCR fingerprints produced after digestion of the same 18 fragments with *Hha*I.

*tococcus* strains were mapped in this manner, using the primer pairs 5.8S-R/LR-1, 5.8S-R/LR-3, 5.8S-R/LR-5, 5.8S-R/LR-6, and 5.8S-R/LR7. The positions of restriction sites in several strains could also be inferred from the additivity of pairs of smaller restriction fragments relative to the larger restriction fragments present in another strain.

**Estimation of genetic relationships between taxa and strains.** For estimating genetic relationships between different rDNA sequences, a distance metric ($D$) was calculated by using the equation suggested by Nei and Li (15), $D = 1 - 2(n_{xy})/(n_x + n_y)$, in which $n_{xy}$ is the number of restriction fragments shared in common between two strains and $n_x$ and $n_y$ represent the total number of restriction fragments in each strain. Two important assumptions regarding $D$ as an estimate of genetic similarity are (i) identity of comigrating restriction fragments from different strains and (ii) the absence of significant length variation within the rDNA region being analyzed (which would result in violation of assumption i).

Estimates of overall restriction site similarity were used to construct an unrooted tree (network) by the FITCH option of the computer package PHYLIP (4).

## RESULTS

**Amplification of rDNA from *Cryptococcus* spp.** The locations of primer sites and strategies for PCR amplification of rDNA from fungi are shown in Fig. 1. These oligonucleotide primers themselves represent highly conserved consensus regions between published rRNA sequences available through GenBank for several fungi and eucaryotes, including *Saccharomyces cerevisiae* (yeast), *Neurospora crassa*, *Oryza sativa* (rice), *Mus domesticus* (mouse), and humans (Table 1). When small differences in the primer sites were found (mostly base substitutions), the yeast sequence was used for primer synthesis. These primers have been successfully used in our laboratory to amplify rDNAs from a variety of fungi, plants, and animals (unpublished data).

Six strains of *Cryptococcus* and *Candida* spp. were chosen initially to amplify three portions of the rDNA repeat (A, B, and C in Fig. 1) spanning almost the entire rDNA coding region. Figure 2A shows samples of six C fragments obtained after PCR amplification from all six strains. All three fragments (A, B, and C) were successfully amplified from all strains tested, with a typical yield of 1 to 2 μg per 100-μl

reaction. The lengths of the A and C fragments were 2.0 and 1.6 kilobase pairs (kb), respectively, in all six strains. Minor length heterogeneity was observed for the B fragments from *C. neoformans* 132 and n32 (1.85 kb), *Cryptococcus albidus* 142 (1.85 kb), *Cryptococcus uniguttulatus* 103.87 (1.80 kb), *Cryptococcus laurentii* 139 (1.80 kb), and *Candida albicans* 666 (1.75 kb).

**RFLPs between species.** Samples of the A, B, and C fragments were digested with either *Hha*I or *Ava*II and resolved by electrophoresis in 3% agarose. Restriction digests for each set of digested PCR fragments yielded similar patterns of variation among the six strains tested (Fig. 2B; only the *Hha*I-digested C fragments are shown). We have occasionally observed partial digestion of PCR fragments with use of some enzymes, suggested by the presence of secondary bands of lighter intensity in the photos. The primary restriction patterns are clearly obvious in most cases, however, and allow unambiguous assignment of strains to a particular restriction phenotype class. Both *C. neoformans* strains (representing the two varieties, *neoformans* and *gattii*) produce identical restriction patterns with either enzyme for all three fragments. *C. albidus* 142 and *C. uniguttulatum* 103.87 shared identical restriction patterns for two of the six enzyme-fragment combinations tested. *C. laurentii* 139 and *Candida albicans* 666 each produced unique restriction patterns for each fragment with use of either enzyme.

The rDNA region corresponding to fragment B (Fig. 1) was chosen for more detailed and extensive analysis using an expanded set of isolates. Purified B fragments were amplified by PCR from 16 reference strains (including several type strains) representing *Cryptococcus* taxa known to be pathogenic. DNAs from two strains of *Candida albicans* were also included in the experiment as taxonomically unrelated controls. This collection of B fragments was digested with four separate enzymes (*Taq*I, *Ava*II, *Hha*I, and *Hin*fI). Photographs of the *Ava*II and *Hha*I digests are shown in Fig. 3. The restriction phenotypes identified for the various enzyme-strain combinations are listed in Table 3. A pattern of taxon-specific restriction phenotypes (i.e., fingerprints) was clearly evident for all four enzymes examined. A fifth enzyme, *Msp*I, also showed similar taxon-specific variation but was not surveyed in all strains (data not shown). All of the *C. neoformans* isolates had identical PCR fingerprints for all four enzymes. Similarly, both strains of *C. uniguttulatus* had identical restriction phenotypes for every enzyme surveyed. Intraspecies polymorphism was evident among several type strains representing different varieties of *C. albidus* as well as between the two strains of *Candida albicans*. By scoring the 18 strains for their restriction patterns produced with each enzyme, 10 unique genotypic classes could be identified (Table 3). All of the *C. neoformans* strains, including its two varieties *gattii* and *neoformans* (8, 10), had identical PCR fingerprints for each enzyme tested (class I in Table 3). Both strains of *C. uniguttulatum* were also identical for all four enzymes (class VI). The type strains of *Cryptococcus ater* (class VII) and *C. laurentii* (class VIII) were each characterized by unique fingerprints. Strains from the remaining taxa were polymorphic for one or more enzymes as follows: several varieties of *C. albidus* comprised classes II, III, IV, and V, and two strains of *Candida albicans* made up classes IX and X.

**Identification of isolates by using PCR fingerprints.** An additional 15 *Cryptococcus* strains representing clinical isolates were tested to determine whether their PCR fingerprints could be assigned to one of the reference classes identified previously. Each of the clinical isolates had been tentatively identified by its source to species on the basis of morphological or cultural features. The B fragments of each strain were amplified and digested with the four enzymes used previously. An example of one of the restriction digests (for *Ava*II) is shown in Fig. 4. Eleven clinical isolates identified as *C. neoformans* (including four strains isolated from patients infected with human immunodeficiency virus) produced PCR fingerprints characteristic of genotypic class I, to which all of the reference strains of *C. neoformans* belong. Four isolates identified as either *C. albidus* (one isolate) or *C. laurentii* (three isolates) produced PCR fingerprints that were similar to some but not all of the previous reference strains. Two of these clinical isolates (*C. laurentii* 318 and *C. laurentii* 108.87) produced unique restriction patterns not encountered in any of the previous reference strains, whereas the other two isolates possessed a unique array of restriction patterns for the four enzymes tested. As a result, it was necessary to assign these four clinical isolates to three additional genotypic classes (XI, XII, and XIII).

**PCR mapping of restriction sites in rDNA.** Figure 3B shows six *Hha*I restriction fragments produced after digestion of the B fragment of *C. neoformans* 132. Figure 5 shows results

TABLE 3. Genotypic classes and restriction phenotypes of *Cryptococcus* and *Candida* spp. revealed by PCR fingerprints

| Class | Strain | Restriction phenotype of B fragment digested with: | | | |
|---|---|---|---|---|---|
| | | *Taq*I | *Hin*fI | *Ava*II | *Hha*I |
| | Reference strains | | | | |
| I | *C. neoformans* 132[a] | A | A | A | A |
| I | *C. neoformans* 3501 | A | A | A | A |
| I | *C. neoformans* 3502 | A | A | A | A |
| I | *C. neoformans* 101 | A | A | A | A |
| I | *C. neoformans* C3D | A | A | A | A |
| I | *C. neoformans* n33 | A | A | A | A |
| I | *C. neoformans* n34 | A | A | A | A |
| I | *C. neoformans* n32 | A | A | A | A |
| II | *C. albidus* var. *aerius* 155[a] | B | B | B | B |
| III | *C. albidus* var. *albidus* 142[a] | C | —[b] | B | — |
| IV | *C. albidus* var. *diffluens* 160[a] | D | C | C | C |
| V | *C. albidus* var. *ovalis* K6-7[a] | C | D | D | D |
| VI | *C. uniguttulatus* 103.87 | B | E | E | D |
| VI | *C. uniguttulatus* 162.86 | B | E | E | D |
| VII | *C. ater* 4685[a] | E | F | F | D |
| VIII | *C. laurentii* 139[a] | F | — | G | E |
| IX | *Candida albicans* 666 | G | G | H | F |
| X | *Candida albicans* C9M | H | — | J | — |
| | Clinical and other strains | | | | |
| I | *C. neoformans* n10[b] | A | A | A | A |
| I | *C. neoformans* n11[b] | A | A | A | A |
| I | *C. neoformans* n12[b] | A | A | A | A |
| I | *C. neoformans* n31[b] | A | A | A | A |
| I | *C. neoformans* n16 | A | A | A | A |
| I | *C. neoformans* n18 | A | A | A | A |
| I | *C. neoformans* n25 | A | A | A | A |
| I | *C. neoformans* n27 | A | A | A | A |
| I | *C. neoformans* 602 | A | A | A | A |
| I | *C. neoformans* D321 | A | A | A | A |
| I | *C. neoformans* n35 | A | A | A | A |
| XI | *C. albidus* D322 | B | D | B | D |
| XII | *C. laurentii* D318 | F | — | A | — |
| XIII | *C. laurentii* 108.87 | C | I | K | G |
| XI | *C. laurentii* 142.89 | B | D | B | D |

[a] Type culture for species or variety.
[b] —, Restriction phenotype not determined.
[c] Isolate from patient with acquired immunodeficiency syndrome.

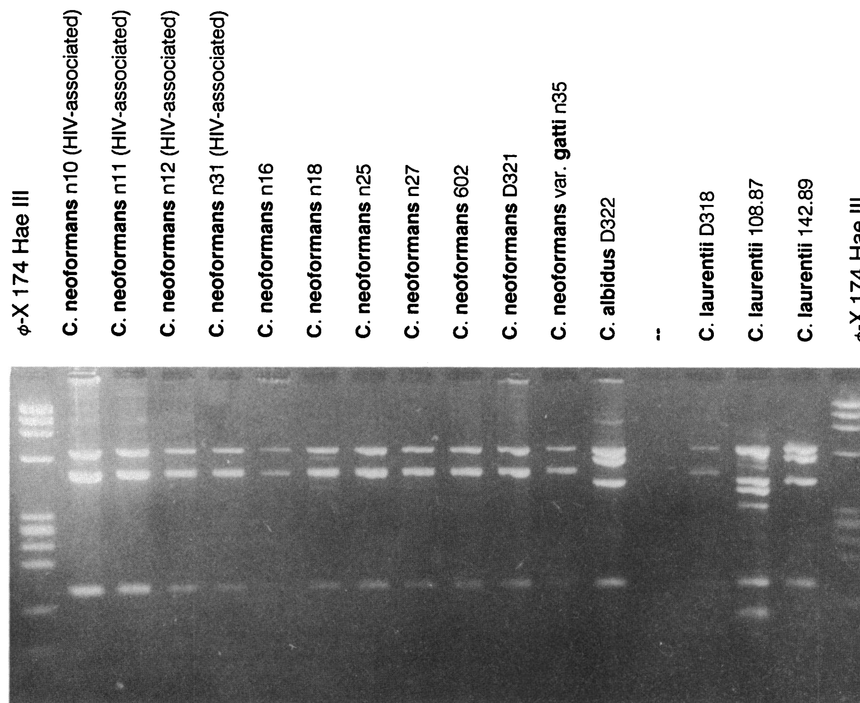


FIG. 4. PCR fingerprinting used to identify taxonomic identity of 15 *Cryptococcus* strains. Enzymatically amplified rDNA (fragment B) from each strain was digested with *Ava*II and analyzed in 3% agarose. Eleven clinical isolates of *C. neoformans* produce identical restriction phenotypes and belong to a single genotypic class. Four remaining strains have fingerprints that are either unique or characteristic of other genotypic classes.

of a mapping experiment used to determine the positions of the six *Hha*I fragments from this strain. Five incremental PCR fragments ranging from 350 to 1,750 base pairs (bp) were amplified (Fig. 5A) and digested simultaneously with *Hha*I. The resulting restriction fragments were run adjacently on a 3% agarose gel (Fig. 5B). The smallest restriction fragment (50 bp, indicated by an arrow) is present in all of the incremental PCR products, indicating its location within 5′ end of the B fragment. The large 550-bp restriction fragment appears in the next and all subsequent lanes, and so its position must be next after the 50-bp fragment. In a similar fashion, the positions of the remaining four restriction fragments of the B fragment can be determined by their serial appearance from left to right in the five restriction digests. The resulting map positions of the *Hha*I restriction sites within the B fragment of *C. neoformans* 132 are shown in Fig. 6, along with sites for *Ava*II, *Taq*I, and the PCR primers themselves (LR1 through LR7). Restriction maps of all of the *C. neoformans* strains were identical for these three enzymes. Additional restriction site differences between *C. neoformans* and the other *Cryptococcus* strains were determined by PCR mapping or by inferring site changes on the basis of different restriction patterns, indicated below each map in Fig. 6. The order of several smaller restriction fragments generated by *Taq*I, as well as the numerous *Hinf*I fragments present in most strains, could not be mapped accurately because of limited resolution of 3% agarose gels for fragments of less than 50 bp. Some strains of *C. albidus* and *C. laurentii* also yielded an apparent excess number of restriction fragments that could not be mapped unequivocally, since their summed sizes exceeded the total length of the B fragment. We suspect that the presence of additional fragments in some strains may be due to minor sequence

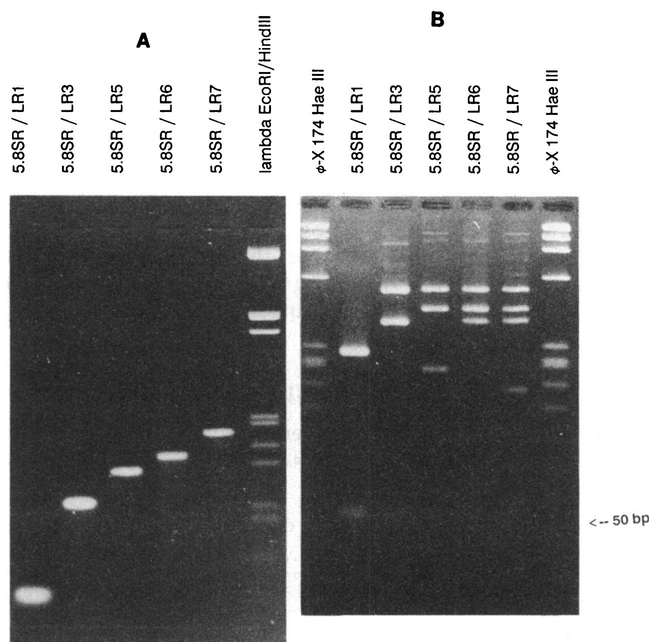


FIG. 5. PCR-assisted mapping of restriction sites (PCR mapping) in *C. neoformans* 132. (A) Fragment ladder produced after PCR amplification of five incrementally larger fragments of rDNA (fragment B), starting from a common endpoint; (B) restriction patterns of the same five PCR fragments digested simultaneously with *Hha*I. The order of restriction fragments in the largest fragment (5) can be determined by their serial appearance from left to right in each of the five lanes. The position of a faint 50-bp fragment present in all lanes is indicated by the arrow.

FIG. 6. Partial restriction maps for one region of the rDNA repeat (fragment B) from *C. neoformans* 132 and other *Cryptococcus* spp. Locations of conserved restriction sites present in all strains are shown above the lines; variable sites are indicated below with the respective genotypic class.

heterogeneity among the numerous rDNA repeats that exist as a multigene family.

**Genetic relationships between rDNAs from different *Cryptococcus* spp.** Differences in the number of common restriction fragments among PCR fingerprints (Fig. 3 and 4) suggest that some strains may be closely related. To estimate the level of divergence between PCR-amplified rDNA fragments from different strains, a matrix of pairwise genetic distances was calculated for the genotypic classes defined in Table 3 (restriction patterns of the two *Candida albicans* strains shared little or no similarity with each other and with the other strains and were therefore not included in this analysis). The distance matrix (Table 4) was used to construct a branching network based on the Fitch-Margoliash algorithm, using the FITCH routine of the PHYLIP computer package (Fig. 7). This unrooted network divides the strains into roughly three equidistant groups, with all of the *C. neoformans* strains at one end and most of the other taxa placed on numerous terminal branches at the opposite end. Two strains of *C. laurentii* belonging to genotypic classes VIII and XII were placed roughly equidistant between the two groups (Fig. 7).

FIG. 7. Genetic relationships among different rDNA genotypic classes identified by PCR fingerprinting. A distance matrix based on restriction fragment analyses of rDNA fragment B (Fig. 1) was used to construct an unrooted network, using the FITCH procedure of PHYLIP.

## DISCUSSION

**Analysis of rDNA from pathogenic fungi by using the PCR.** Successful amplification of rDNA was obtained for every strain of *Cryptococcus* and *Candida* spp. tested by using the PCR primers listed in Table 2. We have successfully used these primers to amplify rDNA in numerous other fungi, including ascomycotina and basidiomycotina (data not shown). By using the same primer sequences or their complementary strands, amplification of other rDNA regions should also be attainable (e.g., the nontranscribed spacer region). The ability of the PCR to amplify known variable regions of DNA, as well as more conserved tracts, should permit analysis at almost any organismal level, from individual strains to higher taxa.

The method for extraction of genomic DNA from yeast cells had little apparent effect on the yield and quality of PCR products. Purified template DNA was not found to be necessary for efficient amplification, which permitted the use of simplified extraction protocols. We found it convenient to gel purify DNA minipreps before use by running a portion of each sample into low-melting-point agarose, followed by dilution of the excised genomic DNA band into 1 ml of distilled water. This procedure effectively removed potential inhibitors in every case. Precise quantitation of DNA in these dilutions was not required, the primary criterion being the presence of a visible high-molecular-weight DNA band in the gel.

**PCR fingerprinting for identification of *Cryptococcus* species and varieties.** Analysis of restriction patterns for three separate regions of the rDNA repeat (A, B, and C in Fig. 1) revealed the presence of considerable sequence variation among the strains studied and provides a convenient method for fingerprinting of species, varieties, and even individual strains. Using four restriction enzymes with 4-base recognition sequences, we were able to recognize 13 distinct geno-

TABLE 4. Genetic distance matrix among 11 genotypic classes of *Cryptococcus* spp. based on restriction pattern analysis of rDNA fragment B

| | II | I | III | IV | V | VI | VII | VIII | XI | XII |
|---|---|---|---|---|---|---|---|---|---|---|
| XII | 0.532 | 0.362 | 0.333 | 0.370 | 0.362 | 0.447 | 0.469 | 0.455 | 0.333 | 0.400 |
| XII | 0.294 | 0.333 | 0.400 | 0.300 | 0.333 | 0.294 | 0.333 | 0.111 | 0.333 | |
| XI | 0.535 | 0.256 | 0.182 | 0.200 | 0.070 | 0.209 | 0.289 | 0.400 | | |
| VIII | 0.263 | 0.400 | 0.455 | 0.364 | 0.400 | 0.368 | 0.300 | | | |
| VII | 0.545 | 0.364 | 0.455 | 0.294 | 0.318 | 0.136 | | | | |
| VI | 0.571 | 0.286 | 0.333 | 0.265 | 0.238 | | | | | |
| V | 0.571 | 0.286 | 0.182 | 0.224 | | | | | | |
| IV | 0.592 | 0.306 | 0.333 | | | | | | | |
| III | 0.524 | 0.182 | | | | | | | | |
| II | 0.429 | | | | | | | | | |

typic classes among the 33 strains surveyed (Table 3). By amplifying a relatively large PCR fragment (≈1 to 2 kb) from several strains, genetic differences can be easily determined by digesting PCR products with different frequent-cutting enzymes. Subsequent blotting and probing with labeled DNA probes is not necessary, and the restriction patterns are directly observable during electrophoresis. Another significant advantage to using PCR-generated fragments for restriction analysis is the avoidance of poor digestion of genomic DNA by some restriction endonucleases caused by DNA base modification (i.e., methylation).

Although we were able to detect genetic variation among most *Cryptococcus* species and strains from this study, no differences were observed among the 19 strains of *C. neoformans* with use of the four restriction enzymes. Genetic uniformity within *C. neoformans* was unexpected, since our strains represent two well-characterized varieties (*gattii* and *neoformans*) that differ in a number of biologically significant features, including serotype, colony morphology, nutrition, and DNA-DNA hybridization (1, 3, 9, 10). By surveying other regions of the genome or using a wider array of restriction enzymes, it should be possible to identify diagnostic PCR fingerprints among the varieties and serotypes of *C. neoformans* as well. We have obtained preliminary data indicating that the two *C. neoformans* varieties differ by an *AluI* polymorphism within the B fragment (unpublished data).

Considerable variability was observed among PCR fingerprints in the remaining 12 *Cryptococcus* and two *Candida* strains examined; these variations could be grouped into 12 genotypic classes (Table 3). Although we did not attempt to characterize genetic differences between the two strains of *Candida albicans* from this study, inspection of their respective PCR fingerprints suggests that the two strains probably differ by length mutations rather than any specific restriction site loss or gain. A recent survey of rDNA polymorphism in *Candida* spp. also demonstrated considerable length heterogeneity in rDNA among strains of *C. albicans* (13). By using the PCR approach described here, it ought to be possible to rapidly identify taxa and strains of *Candida* spp. as well as of other medically important fungi.

Type strains of all four varieties of *C. albidus* were each characterized by a unique combination of restriction patterns with use of the four enzymes. Each type strain of *C. ater* and *C. laurentii* was also assignable to its own unique genotypic class. Although additional evidence is required before any taxonomic conclusions can be reached, it appears that *C. albidus* and its varieties, as well as *C. laurentii*, are genetically variable as currently circumscribed.

**Linking of sexual and asexual life histories and the genetic relationships of *Cryptococcus* spp.** One powerful application of PCR fingerprints is for establishing biological identity between asexual and sexual stages of the fungal life cycle. The complex life cycles of many fungi such as *Cryptococcus* spp. have resulted in separate teleomorphic (sexual) and anamorphic (asexual) taxonomic systems that are not well integrated. Of the 19 *C. neoformans* strains that we surveyed, only several have been induced to produce a *Filobasidiella* teleomorph in culture. The uniformity of genotypic class I based on PCR fingerprint analysis strongly suggests an association for all of these strains with the genus *Filobasidiella*. In a similar fashion, a direct relationship between most of the other *Cryptococcus* strains and the basidiomycete genus *Filobasidium* is suggested by their PCR fingerprints. A high level of genetic relatedness is indicated in Fig. 7 for different strains in the *C. albidus* group, which includes strains known to have a *Filobasidium* teleomorph. Strains associated with this *Filobasidium* complex include *C. ater* and *C. uniguttulatus* as well as two clinical isolates identified as *C. laurentii*. Of these three taxa, only *C. uniguttulatus* has previously been shown to have a teleomorph in the genus *Filobasidium* (10).

The analysis of genetic distances indicated by PCR fingerprinting grouped the 11 genotypic classes into three major groups (Fig. 7). The most distant grouping includes all of the *C. neoformans* strains belonging to genotypic class I. This arrangement supports previous conclusions that *C. neoformans* and its *Filobasidiella* teleomorph are phylogenetically unique from other taxa in the family *Filobasidiaceae* (7, 9, 10).

Most of the remaining *Cryptococcus* strains belong to a genetically variable group characterized by a *Filobasidium* teleomorph, which includes the different varieties of *C. albidus* as well as *C. ater* and *C. uniguttulatus*. The genetic relationship of *C. laurentii* with the other *Cryptococcus* spp. is less certain. Two strains identified as *C. laurentii* (genotypic classes XI and XIII) clearly belong in the *Filobasidium* group identified above. Two remaining strains of *C. laurentii* (classes VIII and XII, including the type strain) occupy an intermediate position in Fig. 7 between the *C. neoformans* group representing the genus *Filobasidiella* and the *Filobasidium* group, which includes *C. albidus*. These observations are intriguing, since *C. laurentii* has been taxonomically distinguished from the *C. albidus* group by its inability to assimilate nitrate and the fact that it is not associated with a known teleomorph. Although nitrate assimilation has been widely used in yeast systematics, its value as a phylogenetically informative character has recently come under question (5). This apparent genetic relatedness of certain *C. laurentii* strains with *C. albidus* warrants further scrutiny of nitrate assimilation as a taxonomic character in this group.

Further study of sequence variation within the rDNAs of these strains should also be useful for understanding the evolutionary history of these fungi. The genetic relationships indicated in Fig. 7 therefore also represent a phylogenetic hypothesis. Additional study using outgroup taxa as well as a larger set of strains should aid in resolving the proposed phylogeny further. Sequence data obtained by using the PCR (2, 19, 22) ought to be especially valuable in this regard.

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