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# New PCR Primer Pairs Specific for *Cryptococcus neoformans* Serotype A or B Prepared on the Basis of Random Amplified Polymorphic DNA Fingerprint Pattern Analyses

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**Thirty-three strains of *Cryptococcus neoformans* were isolated from clinical specimens, including specimens from AIDS patients in Brazil, and were classified into two serotypes; we detected 31 and 2 strains of serotypes A and B, respectively. Random amplified polymorphic DNA (RAPD) fingerprint pattern analyses of these strains of serotypes A and B showed that the patterns were similar for strains of each serotype when three 10-mer primers were used as the RAPD primers. Comparative studies of the fingerprint patterns of the study isolates with those of the reference strains also showed that the RAPD patterns for strains of each serotype were related and that most of the fingerprint bands existed commonly for all strains of each serotype tested. The common RAPD bands (an approximately 700-bp band for serotype A and an approximately 450-bp band for serotype B) were extracted and the DNA sequences were determined. Using this information, we prepared two and one PCR primer pairs which were expected to be specific for *C. neoformans* serotypes A and B, respectively. Use of each PCR primer combination thus prepared for serotype A or B was 100% successful in identifying the respective *C. neoformans* serotypes, including the 33 clinical isolates tested in the present study. Among these combinations, one for serotype A was found to amplify DNA from *C. neoformans* serotype B as well as serotype A. Serotype B-specific PCR primer pairs amplified DNA from not only serotype B strains but also from serotype C strains. The usefulness of other serotype-specific PCR primers for clinical *C. neoformans* isolates is discussed.**

*Cryptococcus neoformans* is a cosmopolitan fungus and causes cryptococcosis in humans and animals (7, 8). Recent increases in the numbers of AIDS patients in Brazil have also led to increases in the numbers of infections caused by this pathogenic fungus (5).

On the basis of the antigenic composition of its polysaccharide capsule and biological differences, *C. neoformans* has been subdivided into two varieties and five serotypes, namely, *C. neoformans* var. *gattii* (serotypes B and C) and *C. neoformans* var. *neoformans* (serotypes A, D, and AD). It has been reported that the isolates of *C. neoformans* that infect AIDS patients are predominantly of serotype A (7–9), and we also confirmed these findings of the predominance of serotype A by epidemiological studies with AIDS patients in Thailand (14). Isolation of a serotype D strain from AIDS patients has also been reported in France, Germany, and the United States (3).

Several typing approaches have been used in epidemiological studies, including serotyping and PCR fingerprinting (2, 6, 11). Among them, the serotyping schemes have been found to be important since the association of infection with serotype A strains in AIDS patients was confirmed by recent epidemiological and ecological studies (4, 10, 20). Random amplified polymorphic DNA (RAPD) fingerprinting has been demonstrated to have the ability to discriminate between closely related isolates within a given population (17, 18, 21). Ruma et al. (15) reported that RAPD fingerprinting techniques are useful for distinguishing among the varieties and serotypes of *C. neoformans*, and Varma and Kwong-Chung (19) reported on similar results obtained with various RAPD primers. No useful PCR primers derived from published RAPD analyses of *C. neoformans* serotypes have been reported.

In the study described here, we developed new, useful PCR primer pairs which should be specific for *C. neoformans* serotype A or B on the basis of RAPD fingerprint pattern analyses of *C. neoformans*. The usefulness of other *C. neoformans* serotype-specific PCR primer pairs, such as primer pairs specific for serotypes C, D, and AD, for the identification of *C. neoformans* strains is also discussed.

## MATERIALS AND METHODS

**Microorganisms and determination of serotypes of *C. neoformans* strains.** The following isolates of *C. neoformans* were used as reference strains: for serotype A, strain CDC 551; for serotype B, strain NIH 112; for serotype C, strain NIH 18; for serotype D, strain IFM 5857; and for serotype AD, strain LY 23 (an isolate from China). Thirty-three clinical *C. neoformans* isolates from the State University of Campinas were used. The fungal strains were inoculated onto potato dextrose agar (Difco) slants and were incubated at 30°C for approximately 48 to 72 h. After two transfers, the serotype of each strain was determined by slide

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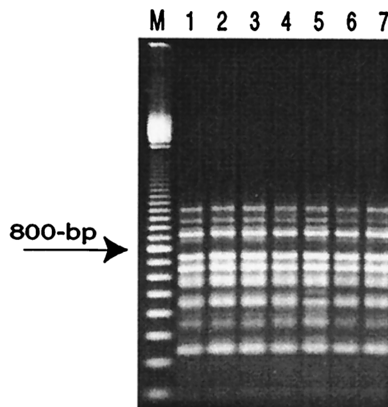


FIG. 1. RAPD fingerprint patterns of seven strains of *C. neoformans* serotype A isolated in Brazil obtained with primer R-1. Lane M, DNA ladder marker used as the molecular size standard; lanes 1 to 7, clinical serotype A isolates from Brazil.

agglutination tests (Crypto Check; Iatron Co., Tokyo, Japan), and the serotypes were compared with those of the reference strains of *C. neoformans* (14).

**Extraction of DNA.** The chromosomal DNAs of all *C. neoformans* strains were extracted by a method modified from those of Zhu et al. (22) and Poonwan et al. (14). The strains were grown on brain heart infusion agar at 37°C for 1 week. Three or four loopfuls of the fungal yeast cells from the agar slants were suspended in 500 μl of TE buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA) in an Eppendorf tube (1.5 ml) and then kept in a heater at 80°C for 30 min to kill the fungus. After shaking, the suspension was centrifuged at 11,000 × *g* and the sediment was mixed with 0.5 ml of extraction buffer (100 mM Tris-HCl [pH 9.0] with 40 mM EDTA), 0.1 ml of 10% sodium dodecyl sulfate, and 0.3 ml of benzyl chloride. After shaking with a vortex mixer, the fungal cells were incubated at 60°C for 30 min and then broken with a bead beater (Wakenyaku Co., Kyoto, Japan) for 20 s. After centrifugation at 11,000 × *g* at 4°C for 5 min, the supernatant was transferred to another Eppendorf tube, 3 M sodium acetate was added at 1/10 volume of the aqueous supernatant, and the mixture was cooled at 0°C for 10 min. The DNA was precipitated with 0.5 ml of 2-propanol (−70°C, 3 h), and then the precipitate was washed with 70% ethanol, dried, and resuspended in 100 μl of TE buffer. The DNA concentration was determined with a UV monitor (Bio-mini UV monitor; ATTO, Tokyo, Japan).

**RAPD PCR analyses.** The following three primers were used for the RAPD analysis: R-1 (5′-ATGGATCGGC-3′), R-2 (5′-ATTGCGTCCA-3′), and R-3 (5′-TCACGATGCA-3′). These were prepared on the basis of the reports of Goodwin and Annis (6) or on the basis of information from our preliminary experiments. Amplification reactions were performed in a volume of 30 μl of distilled water containing 2.5 μl of primer (20 pM), 2.5 μl of genomic DNA (1 μg/ml), and one PCR bead (Ready-to-Go PCR bead; Pharmacia Biotech). The PCR was performed by initially heating the samples at 94°C for 4 min; this was followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 32°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min in a thermoreactor. All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1× TBE (Tris-borate-EDTA) buffer at 80 V for 90 min and then stained in a solution of ethidium bromide at 0.5 μg/ml.

**Extraction and sequencing of DNA from RAPD fingerprint bands.** For extraction and sequencing of DNA from the RAPD fingerprint bands, a RAPD PCR product was subjected to phenol-chloroform extraction and was precipitated with ethanol. The DNA fragments were cloned into the pGEM 3Zf vector and were transformed into bacterial strain JM109 (Technical Manual; Promega Co., Madison, Wis.). Sequencing-grade plasmid DNA was purified with a plasmid purification kit (GFX Micro Plasmid Prep Kit; Pharmacia Biotech). The sequencing was performed on an ABI prism 377A DNA sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Division, Perkin-Elmer Japan Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol.

**Determination of serotypes by PCR with a new PCR primer pair.** The normal PCR with the newly prepared primer sets was performed by initially heating the samples at 94°C for 10 min; this was followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final 10-min extension step at 72°C. All the reaction products were analyzed by the same methods described above for analysis of the RAPD fingerprint patterns.

## RESULTS

**Serotyping and RAPD analyses of clinical *C. neoformans* isolates in Brazil.** During 1996 and early 1998, we isolated 33 strains of *C. neoformans* from patients in the hospital of the State University of Campinas, Campinas, Brazil. Each isolate was classified into one of two serotypes, and there were 31 and 2 strains of serotypes A and B, respectively. No serotype C, D, or AD strain was observed.

The RAPD fingerprint patterns of 31 strains of *C. neoformans* serotype A obtained with three different 10-mer primers were analyzed. The fingerprint patterns of 31 strains of *C. neoformans* serotype A isolated in Brazil were shown to be similar to each other when we used the three 10-mer PCR primers. The RAPD patterns for seven representative strains obtained with the R-1 primer are shown in Fig. 1. We compared these fingerprint patterns for the Brazilian isolates with those for reference serotype A strains and found that they were also similar to each other and that most of the bands were common for each serotype A strain tested, although some minor variations were observed (data not shown).

When we used three different 10-mer primers for RAPD amplification of *C. neoformans* serotype B, each primer also showed a characteristic RAPD fingerprint pattern (data not shown).

Therefore, we were interested in the RAPD patterns of the reference *C. neoformans* serotype A, B, C, D, and AD strains. When we compared the patterns of these reference strains obtained with the three 10-mer primers, we found that the pattern obtained with each primer was serotype specific. Among the three primers, since primer R-2 was found to produce on the electrophoresis gel PCR bands that were sharper than those produced by the other two primers, it was selected for use in the following studies for sequence determination. The RAPD patterns of the reference serotype A, B, C, D, and AD strains obtained with primer R-2 are shown in Fig. 2, and the DNA bands that we used to determine the sequence are indicated by arrows.

**Sequence determination and preparation of new PCR primers.** For the preparation of serotype-specific PCR primers for the detection of *C. neoformans* serotypes A and B, bands of about 700 and 450 bp, respectively, were extracted. After purification of the DNA fragments, they were cloned into the pGEM 3Zf vector, and the sequences were determined with an automated DNA sequencer as described in Materials and Methods. The DNA sequences of the extracted bands are presented in Fig. 3.

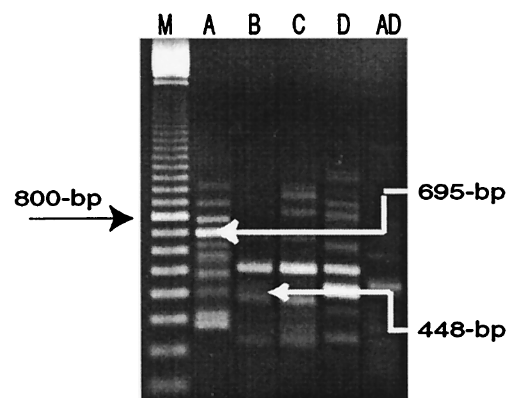


FIG. 2. RAPD fingerprint patterns of the reference *C. neoformans* serotype A, B, C, D and AD strains obtained with primer R-2. Lane M, marker; lanes A to AD, reference serotype A, B, C, D, and AD strains, respectively. The reference strains were CDC 551 (serotype A), NIH 112 (serotype B), NIH 18 (serotype C), IFM 5857 (serotype D), and LY 23 (serotype AD). The arrows indicate the DNA bands that were obtained by RAPD analysis and that were extracted and sequenced in the present experiment.

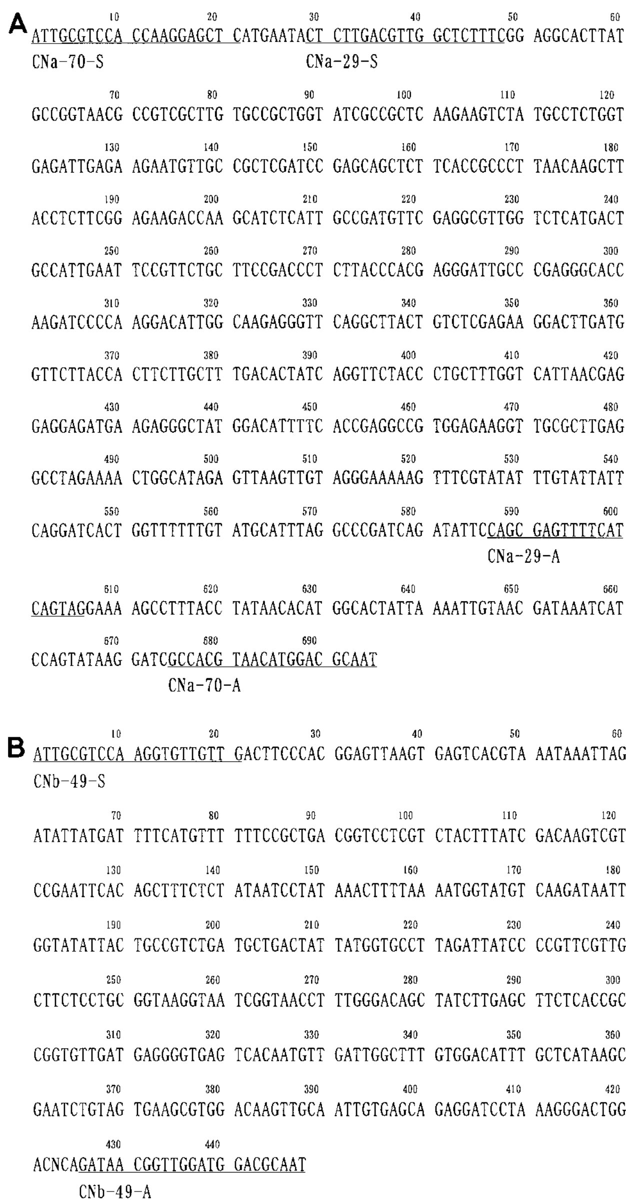


FIG. 3. Newly determined sequence of 695-bp band of DNA from a *C. neoformans* serotype A strain obtained by RAPD analysis and the sequences (underlined) of the new PCR primer pair that was prepared. Newly determined sequence of 448-bp band of DNA from a *C. neoformans* serotype B strain obtained by RAPD analysis and the sequences (underlined) of the new PCR primer pair that was prepared.

TABLE 1. PCR primers prepared on the basis of the DNA sequence information for the RAPD fingerprint pattern bands

| Serotype, PCR primer pair (base sequence) | Origin of DNA (RAPD band size) |
|---|---|
| Serotype A | |
| CNa-70-S (5′-ATTGCGTCCACCAAGGAGCTC-3′) CNa-70-A (5′-ATTGCGTCCATGTTACGTGGC-3′) | *C. neoformans* serotype A (695 bp) |
| Serotype A | |
| CNa-29-S (5′-CTCTTGACGTTGGCTCTTTC-3′) CNa-29-A (5′-CTACTGATGAAAACTCGCTG-3′) | *C. neoformans* serotype A (695 bp) |
| Serotype B | |
| CNb-49-S (5′-ATTGCGTCCAAGGTGTTGTTG-3′) CNb-49-A (5′-ATTGCGTCCATCCAACCGTTATC-3′) | *C. neoformans* serotype B (448 bp) |

**Preparation of new PCR primers for detection of serotype A.** The sizes of the DNA bands for the detection of serotypes A and B were 695 and 448 bp, respectively (Fig. 3A and B). On the basis of the DNA sequence information, two PCR primer pairs believed to be specific for *C. neoformans* serotype A and to amplify the 695-bp fragment (the CNa-70-Sand CNa-70-A primer pair) and the 579-bp fragment (the CNa-29-S and CNa-29-A primer pair) were prepared (Tables 1 and 2).

As shown in Fig. 4, PCR with the CNa-70-S and CNa-70-A primer pair amplified a 695-bp DNA fragment from the reference *C. neoformans* serotype A strain (CDC 551) and three clinical strains of serotype A isolates. The primer pair did not amplify the DNA band from the reference *C. neoformans* serotype B (NIH 112), C (NIH 18), D (IFM 5857), or AD (LY 23) strain when three reference strains were used. When we also tested 31 strains of *C. neoformans* serotype A clinical isolates with this primer pair (CNa-70-S and CNa-70-A), the 695-bp DNA fragment was amplified from all strains (data not shown).

We also tested a PCR primer pair (the CNa-29-S and CNa-29-A primer pair) which was designed to amplify the internal sequence within the 695-bp band. This primer pair amplified a 579-bp fragment of DNA from *C. neoformans* serotype A strains, including 31 of the clinical isolates and the reference strains. There was no amplification of DNA from the reference serotype B, C, D or AD strain (data not shown).

As shown in Fig. 5, PCR with the CNa-29-S and CNa-70-A primer pair amplified a 666-bp fragment of DNA from *C. neoformans* serotype A along with another amplified satellite 460-bp band. When we tested 32 clinical isolates, the primer pair amplified DNA from all *C. neoformans* serotype A strains, which showed 666-bp bands along with the 460-bp satellite band. This PCR primer pair was also found to amplify DNA

TABLE 2. Specificity of newly prepared PCR primer pairs and expected amplified band sizes

| Serotype and PCR primer pair | Expected DNA band size (bp) by PCR | Size (bp) of bands from the following *C. neoformans* strains amplified by PCR: | | |
|---|---|---|---|---|
| | | Serotype A | Serotype B | Serotype C |
| Serotype A | | | | |
| CNa-70-S[a] CNa-70-A[c] | 695[b] | 695 | —[d] | — |
| CNa-29-S CNa-29-A | 579[b] | 579 | — | — |
| CNa-29-S CNa-70-A | 666[b] | 666, 460[e] | 290[e] | 290[e] |
| Serotype B | | | | |
| CNb-49-S CNb-49-A | 448[f] | — | 448 | 448 |

[a] Sense primer.
[b] For serotype A.
[c] Antisense primer.
[d] No amplification.
[e] Estimated DNA band size by agarose gel electrophoresis.
[f] For serotype B.

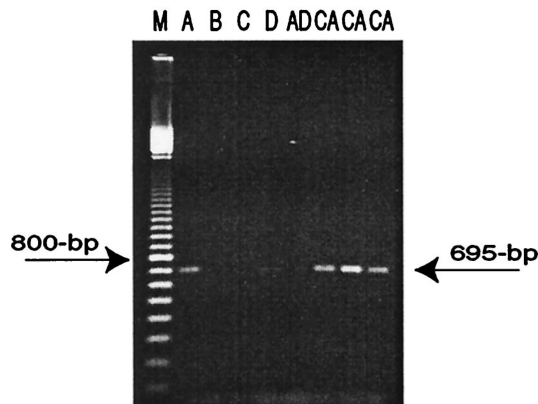


FIG. 4. PCR identification of *C. neoformans* serotype A strains with the CNa-70-S and CNa-70-A PCR primer pair. Lane M, marker; lanes A to AD, reference strains of serotypes A, B, C, D, and AD, respectively; lanes CA, clinical serotype A isolates (CAMP-1, CAMP-2, and CAMP-4 from left to right, respectively) from Brazil.

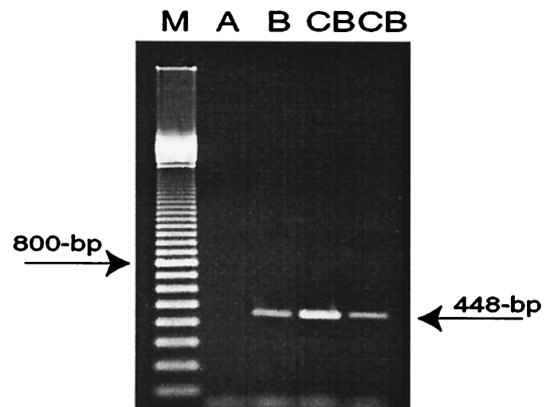


FIG. 6. PCR identification of two clinical strains of *C. neoformans* serotype B with the CNb-49-S and CNb-49-A PCR primer pair. Lane M, marker; lanes A and B, reference strains of serotypes A (CDC 551) and B (NIH 112), respectively; lanes CB, serotype B clinical isolates (CAMP-3 and CAMP-24 on the left and right, respectively) from Brazil.

from *C. neoformans* serotype B and C reference strains, which showed a PCR product of about 290 bp.

**Preparation of new PCR primers for serotype B.** The CNb-49-S and CNb-49-A PCR primer pair (Tables 1 and 2) amplified DNAs from clinical *C. neoformans* serotype B isolates from Brazil (strains CAMP-3 and CAMP-24) and the reference serotype B strains (NIH 112), and amplification of DNA from a *C. neoformans* serotype C strain (strain NIH 18) was also observed (data not shown). The PCR identification of two clinical strains of *C. neoformans* serotype B is shown in Fig. 6. This PCR primer combination was not able to amplify DNA from reference *C. neoformans* serotype A, D, or AD strains.

## DISCUSSION

It has been reported that *C. neoformans* serotype A is distributed worldwide and is a major cause of all clinical types of cryptococcosis (7). *C. neoformans* serotype B, on the other hand, has a more restricted global distribution and is found in subtropical to tropical countries. Since Brazil is located in a

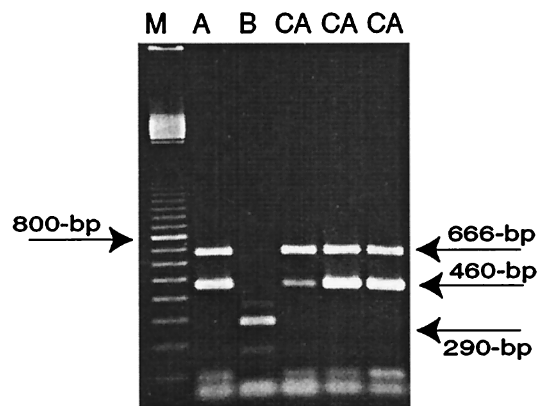


FIG. 5. PCR identification of *C. neoformans* serotype B strains with the serotype A-specific CNa-29-S and CNa-70-A PCR primer pair. Serotype A and B strains were identified by the presence of amplified bands of 666 bp (along with one of 460 bp) and 290 bp, respectively. Lane M, marker; lanes A and B, reference strains of serotypes A (CDC 551) and B (NIH 112), respectively; lanes CA, clinical serotype A isolates (CAMP-1, CAMP-2, and CAMP-4 from left to right, respectively) from Brazil.

tropical region, the existence of cryptococcosis due to serotype B is likely. Kwon-Chung et al. (9) reported that *C. neoformans* var. *neoformans* constituted about 98 to 100% of isolates from AIDS patients in Brazil and Southern California and that 40 to 45% of the isolates from non-AIDS patients were serotype B. Therefore, the present data indicating that 31 of 33 strains were *C. neoformans* serotype A may be reasonable, because most of the present *C. neoformans* strains from Brazil were isolated from AIDS patients.

Molecular techniques such as restriction fragment length polymorphism analysis and RAPD analysis have provided powerful tools for the direct analysis of the genomes and for epidemiological studies of many pathogens (1, 10, 16, 21). Our data showed that the RAPD fingerprint patterns of 31 clinical isolates of *C. neoformans* serotype A are very similar, suggesting that most of the Brazilian *C. neoformans* serotype A isolates were genetically homogeneous, although minor variations were obtained by RAPD analysis with primer R-1 (Fig. 1). The reason for the genetic homogeneity of these Brazilian isolates of *C. neoformans* serotype A by RAPD analyses is not clear, but it may be due to the limited region from which strains were isolated, that is, the State University of Campinas. To confirm this homogeneity of Brazilian isolates of *C. neoformans* serotype A, comparative RAPD studies with different primers and other strains from different regions of Brazil are in progress at the State University of Campinas, Campinas, Brazil.

Ruma et al. (15) reported that RAPD analysis techniques can distinguish between serotype A, D, or AD strains of *C. neoformans* var. *neoformans*. They also reported that separation of serotype B or C *C. neoformans* var. *gattii* strains by the RAPD method is possible. Varma et al. (20) analyzed 156 strains of *C. neoformans* according to their RAPD fingerprint patterns and concluded that *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* can be classified into several groups according to their distinctive DNA fingerprint patterns. Our present RAPD studies also indicated that discrimination of serotypes according to these patterns is plausible.

Although many reports have shown that RAPD pattern analysis is useful for the identification of the serotypes of *C. neoformans*, developmental studies of a *C. neoformans* serotype-specific primer pair that could be useful for PCR have not been documented. In the experiments described here, we prepared PCR primer pairs which were specific for serotype A

or B strains because these were the only two serotypes isolated from the samples from Brazil. On the basis of the information obtained from RAPD analysis, we designed and prepared two and one PCR primer pairs which were expected to be specific for serotypes A and B, respectively. Use of these pairs (CNa-70-A and CNa-70-A, CNa-29-S and CNa-29-A, and CNb-49-S and CNb-49-A) for the identification of serotypes was 100% successful when we tested samples of DNA from 31 strains of *C. neoformans* serotype A and 2 strains of *C. neoformans* serotype B, including each reference strain. To our knowledge, this is the first report of primers that can be used for the serotype determination (serotype A or B) of *C. neoformans* strains by PCR.

Furthermore, among four PCR primers specific for serotype A, a different primer pair (CNa-29-S and CNa-70-A) will be useful for the rapid detection of the two serotypes of fungal pathogens (serotypes A and B) in Brazil. Discrimination of the two serotypes by previously used routine biological methods has not been easy in clinical laboratories in Brazil which handle samples from AIDS patients, and *C. neoformans* serotype A and B strains are major clinical isolates at the hospital of the State University of Campinas. The advantage of using this PCR primer pair is that an experiment in one PCR tube can simultaneously identify serotype B and C strains as well as serotype A strains. It is not clear why the serotype A-specific PCR primer pair (CNa-29-S and CNa-70-A) is able to amplify DNA (about 290 bp) from serotype B or C strains. Since the sequence information for the 290-bp bands is expected to give us useful information, analysis of the sequences is in progress.

Although two separate RAPD fingerprint patterns were recognizable for serotype B and C strains, our present PCR primer pair could not discriminate between the two serotypes. This was a preliminary experiment, but we were successful in preparing two other PCR primer pairs which are expected to be specific for serotype C. These two serotype C-specific PCR primer pairs also amplified DNAs from serotype B strains, however. These data may have some correlation with those in the report of Ruma et al. (15) since they were successful in differentiating serotype B and C strains only with one of seven primers tested, although the differentiation of strains of other serotypes was easier when the strains were tested by RAPD fingerprint pattern analyses. These data are therefore believed to suggest that serotypes B and C are genetically homogeneous.

During the specificity studies, we also found that the CNa-70-S and CNa-70-A PCR primer pair amplified DNA (about 500 bp) from *Cryptococcus albidus*, in addition to *C. neoformans* serotype A. Although more detailed studies are needed, this primer pair should be a promising and useful candidate for the identification of the *C. albidus* fungus, which is sometimes isolated from clinical specimens.

Mitchell et al. (12) reported on a general PCR primer pair (CN4 and CN5) which can identify all varieties of *C. neoformans*, and we confirmed the usefulness of this primer pair for the identification of all serotypes of *C. neoformans*. Although our present PCR primer pairs cannot identify serotype D and AD strains, use of their PCR primer pair combined with the present serotype-specific PCR primer pair may allow the complete differentiation of *C. neoformans* strains into either *C. neoformans* var. *neoformans* or *C. neoformans* var. *gattii*.

Throughout preliminary experiments, we were able to prepare a new PCR primer pair (CNad-4-sense [5′-ATTGCGTC CAGATAACGATTC-3′] and CNad-4-antisense [5′-ATTGC GTCCACACCGCGTGGA-3′] for the detection of serotype AD strains and a primer pair (CNd-2-sense [5′-ATTGCGTC CAGCATACGAATT-3′] and CNd-2-antisense [5′-ATTGCG TCCACATCATGCAGC-3′]) for the detection of serotype D strains on the basis of the band sequence information obtained for each reference serotype strain by RAPD analysis. Each PCR primer pair for the detection of serotypes AD and D was designed to amplify bands of 417 and 244 bp, respectively. Our preliminary experimental results showed that the primer pair specific for serotype AD amplified DNA (417 bp) from *C. neoformans* serotype A, AD, and D reference strains and was not specific for serotype AD. On the other hand, the PCR primer pair for serotype D amplified DNA (244-bp) from the reference serotype D strain but did not amplify DNA from serotype A or AD strains. Interestingly, the PCR primer pair amplified DNA from serotype C and B strains. Since we have only a few *C. neoformans* serotypes AD and D strains in our laboratory, we were unable to clearly determine the specificities of the PCR primer pairs. Detailed studies of this with other several serotype AD and D strains are planned.

The sequences of the 695- and 448-bp fragments seem to be related to certain bacterial and fungal aminotransferases and mammalian and fungal DNA polymerases (as determined with the FAST program) (13). The relation of the supposed serotype specificity of the PCR primer pairs to antigenic differences among capsular polysaccharides of *C. neoformans* is not clear. However, since the subdivision of *C. neoformans* into varieties is not based solely on antigenic determinants of the polysaccharide capsule but is based on biological differences (8, 9, 17), further detailed genetic and biochemical studies are necessary to elucidate the relation of the specific PCR primer and antigenic differences.

## ACKNOWLEDGMENT

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