COMPARISON OF PRIMERS FOR RAPD-PCR FROM ENVIRONMENTAL ISOLATES OF *CRYPTOCOCCUS NEOFORMANS*, *CRYPTOCOCCUS ALBIDUS* AND *CRYPTOCOCCUS LAURENTII* COMPLEX

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ABSTRACT

Various organisms have been characterized by molecular methods, including fungi of the genus *Cryptococcus*. The purposes of this study were: to determine the discriminatory potential of the RAPD (Random Amplified Polymorphic DNA) primers, the pattern of similarity of the *Cryptococcus* species, and discuss their useful application in epidemiological studies. We analyzed 10 isolates of each specie/group: *C. albidus*, *C. laurentii* complex, *C. neoformans* var. *grubii*, all from environmental source, and two ATCC strains, *C. neoformans* var. *grubii* ATCC 90112, and *C. neoformans* var. *neoformans* ATCC 28957 by RAPD-PCR using the primers CAV1, CAV2, ZAP19, ZAP20, OPB11 and SEQ6. The primers showed a good discriminatory power, revealing important differences between them and between species; the SEQ6 primer discriminated a larger number of isolates of three species. Isolates of *C. laurentii* showed greater genetic diversity than other species revealed by all six primers. Isolates of *C. neoformans* were more homogeneous. Only the primer CAV2 showed no amplification of DNA bands for *C. albidus*. It was concluded that the use of limited number of carefully selected primers allowed the discrimination of different isolates, and some primers (e.g., CAV2 for *C. albidus*) may not to be applied to some species.

Key words: *Cryptococcus albidus*; *Cryptococcus laurentii*; RAPD; Molecular Markers.

INTRODUCTION

Fungi of the genus *Cryptococcus* are important agents of infections in immunocompromised individuals, especially those with AIDS. *C. neoformans* and *C. gattii* are the main species involved in cryptococcosis (9, 16), but other species, as *C. laurentii*, *C. albidus*, *C. uniguttulatus*, *C. luteolus*, *C. adeliensis* have been encountered in human and animal infections in recent decades (15).

Typing of *Cryptococcus* isolates, especially *C. neoformans* complex (wich includes *C. neoformans* and *C. gattii*), relies on well established phenotypic characteristics, such as exoenzyme production, serotyping, morphotyping, antifungal susceptibility, killer toxin sensitivity patterns,

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among other (5, 6, 13). However, more sensitive and specific tools, as molecular typing methods, that are able to distinguish subpopulations of the same species of organisms have been developed in recent years. These tools have allowed distinguishing isolates with different profiles of virulence factors, susceptibility to antifungal drugs, and to discriminate strains from distinct geographic areas (12, 28).

Several molecular typing methods, as reliable and practical options, have been studied and improved, although not all methods are equally discriminatory. Randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and more recently, multigene sequence analysis to multilocus sequence typing (MLST) are the most commonly used for the *C. neoformans* complex analysis. Actually, MLST has been purposed as the standardized method for strain typing of *C. neoformans* isolates (17, 21). On the other hand, the RAPD has been one of the most used for typing different microorganisms, such as *Candida albicans* (3, 22). These markers have been applied in detecting polymorphisms, identification and isolation of specific DNA fragments, and have application in genomic analysis of a wide variety of species, with a relatively low cost (27). However, a good typability and accuracy of the method is desirable, so it is important to choose primers that allow a high discriminatory power of the isolates.

The purposes of the present study were to determine the discriminatory potential of the RAPD primers, to analyze the molecular profile of three species of *Cryptococcus* by RAPD-PCR, and to discuss their possible usefulness in epidemiological studies.

MATERIALS AND METHODS

Microorganisms

Ten isolates of each specie/group, *C. albidus* (identified as

CRA01, CRA03, CRA04, CRA06, CRA07, CRA08, CRA10, CRA11, CRA12 and CRA15), *C. laurentii* complex (CRL01, CRL02, CRL04, CRL05, CRL08, CRL09, CRL10, CRL11, CRL12 and CRL13) and *C. neoformans* var. *grubii* (CN13, CN17, CN18, CN19, CN20, CN22, CN23, CN24, CN25, CN26), were obtained from air and avian droppings in public urban areas inhabited by pigeons at the city of Ribeirão Preto, State of São Paulo, Brazil (18). Identification of the isolates was done by classical methods and confirmed by ID32C system (Bio-Merieux, Marcy I'Etoile, France). *C. neoformans* var. *grubii* ATCC 90112 and *C. neoformans* var. *neoformans* ATCC 28957 were included in this study. The strains were stored in both at –20ºC in BHI-glycerol and at 22-28°C in Sabouraud dextrose agar (SDA) subcultured bimonthly.

Genomic DNA extraction and RAPD-PCR

Genomic DNA was extracted as described by Bolano *et al.* (7), with some modifications (19) and stored at –20°C. The reactions were performed using the primers described in Table 1, in a final volume of 25 μL, containing 2.5 μL of 10x enzyme buffer with KCl (Fermentas, Glen Burnie, MD, USA), 3 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTPs, 50 ng of DNA, 0.4 mmol/L of each primer and 1U of Taq DNA polymerase (Fermentas, Glen Burnie, MD, USA). PCR was performed in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). The reactions were as follows: initial denaturation at 94°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by final extension for 10 min at 72°C. Control tubes without genomic DNA were included in each reaction as a control. Amplification products were submitted electrophoresis on 1% agarose gel in TBE buffer 0.5x (89 mmol/L of Tris-borate and 2 mmol/L of EDTA), at 80V for 3 h.

The processed gels were stained with 1 mg/L of ethidium bromide (Invitrogen, São Paulo, SP, Brazil), visualized with UV transilluminator and the images captured by a capture system (Alpha-Innotech, San Leandro, CA, USA). Molecular size standards Lambda-HindIII (Fermentas, Glen Burnie, MD,

USA) were used as reference to band sizes. Only intense and reproducible fragments were considered for analysis.

Reproducibility was assessed by PCR-amplification on two different occasions.

Table 1. Primer sequences for RAPD-PCR.

Primer	Sequence	References
CAV1	5'-CCC GTC AGC A-3'	1, 10
CAV2	5'-AAC GCG CAA C-3'	10
ZAP19	5'-AAG AGC CCG T-3'	4, 8
ZAP20	5'-GCG ATC CCC A-3'	4, 8
OPB11	5'-GTAGACCCGT-3'	14
SEQ6	5'-CCCGTCAGCA-3'	19

The Jaccard coefficient (S_j) was used to calculate the distance between the isolates according to comparison of the bands and their sizes (3). The Multi-variate Statistical Package 3.0 (MVSP 3.0, Kovach Computing Services, Pentraeth, Wales, UK) software was used to analyze the differences. A dendrogram was generated from the data obtained by the cluster method UPGMA (Unweighted Pair Group Method using Arithmetic Averages). A S_j value of 1.00 indicated that the isolates were identical; values between 0.81 and 0.99 represented high similarity, but not identity, and suggest the occurrence of microevolution on a given isolate; values below 0.80 represented unrelated isolates (3, 22).

RESULTS

RAPD patterns for isolates in this study have been translated into the dendrograms (Figures 1 to 3).

C. laurentii complex isolates had the greatest genetic diversity. Identical patterns (S_j = 1.00) were found only with primer CAV1 in a few isolates; three isolates (CRL01, CRL02 and CRL12) constituted one cluster and two isolates (CRL10 and CRL11) another one. All isolates were separated from each other by some other primers, that separated all isolates with

similarities ranging from 0.00 to 0.800. The primer OPB11 showed that CRL09 is not related with any other isolates. Figure 1 shows the dendrogram of *C. laurentii* complex isolates.

C. albidus isolates, in general, showed higher genetic similarity than *C. laurentii* isolates. Only the primer CAV2 did not amplify any DNA bands. Primers ZAP19 and CAV1 formed similar clusters, that had at least seven identical isolates; six groups (S_j = 0.135 to 0.800) were clustered by SEQ6, four of them with a pair of identical isolates (S_j = 1.00). OPB11 grouped six identical isolates (CRA01, CRA06, CRA07, CRA08, CRA10 and CRA11) highly related to two other isolates (CRA03 with S_j = 0.842 and CRA12, S_j = 0.875).

C. neoformans isolates, including ATCC strains, formed three unrelated cluster ($Sj \leq 0.80$), with the primers CAV1, ZAP20 and OPB11, all of them grouping the same isolates, distributed as follows: one cluster with eight identical isolates, the other with three isolates and another one composed by 28957. Primer CAV2 formed two cluster, one formed by 28957 (var. *neoformans*) and the other grouping all identical isolates (all of var. *grubbi*). The primers ZAP19 and SEQ6 (see Figure 4) had higher discriminatory power between the primers tested, forming six and eight groups, respectively.
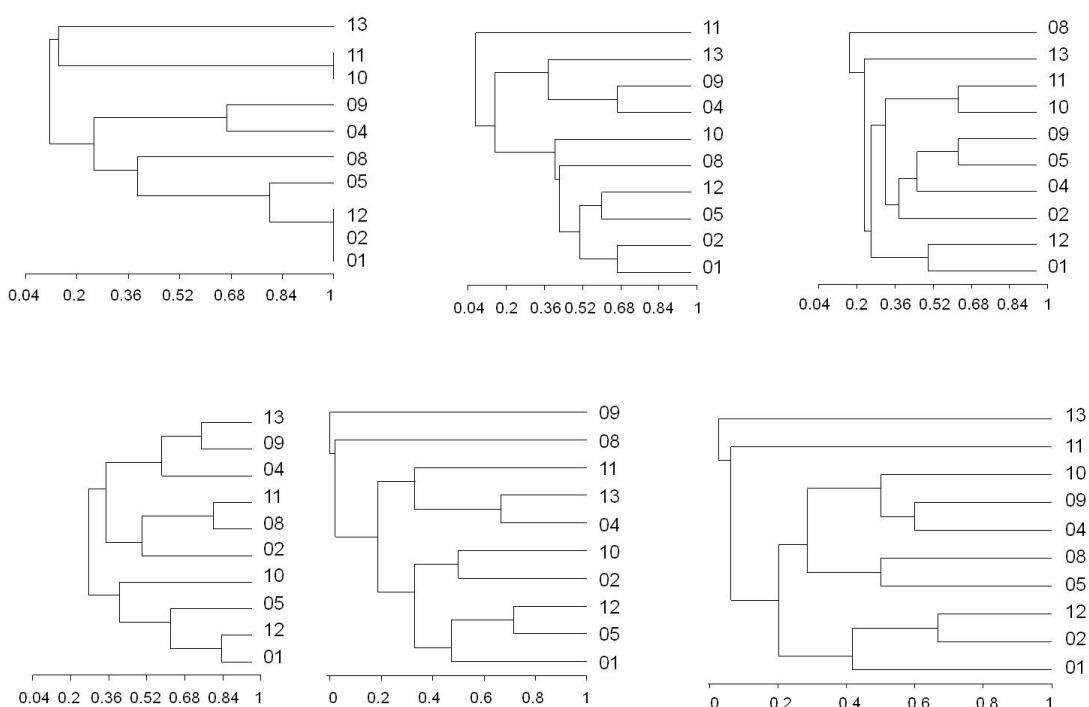
Figure 1. Dendrograms showing the relationships of *C. laurentii* isolates generated from the RAPD-PCR profiles obtained with primers (from left to right) CAV1, CAV2 and ZAP19 (above); ZAP20, OPB11 and SEQ6 (below).
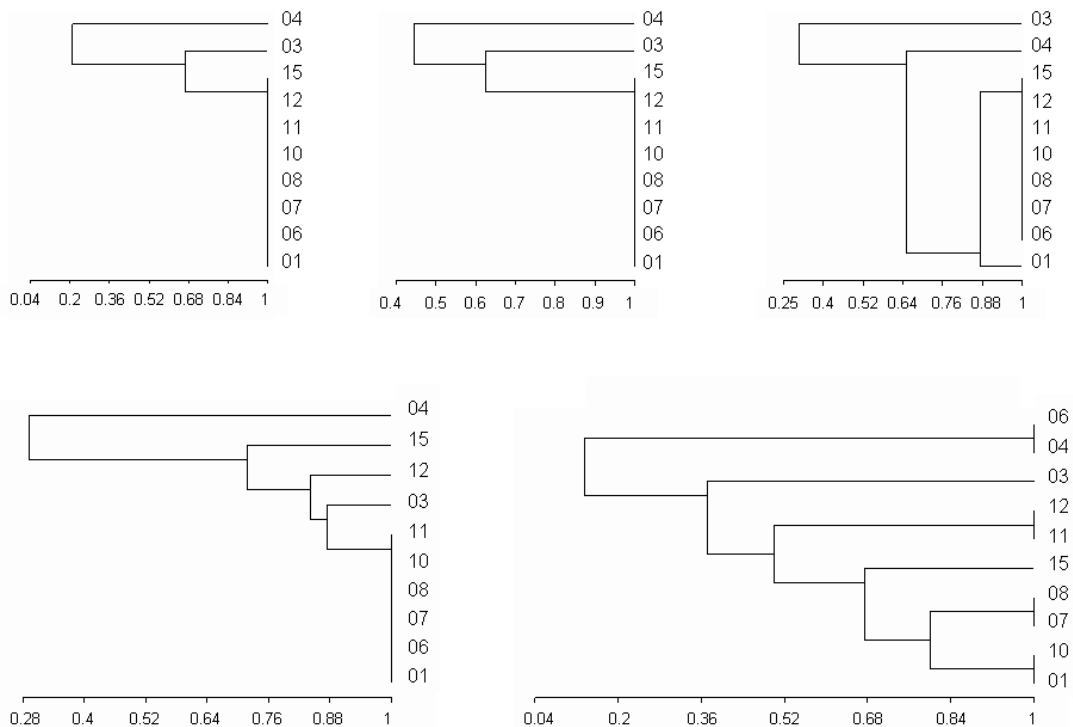

Figure 2. Dendrograms showing the relationships of *C. albidus* isolates generated from the RAPD-PCR profiles obtained with primers (from left to right) CAV1, ZAP19 and ZAP20 (above); OPB11 and SEQ6 (below).
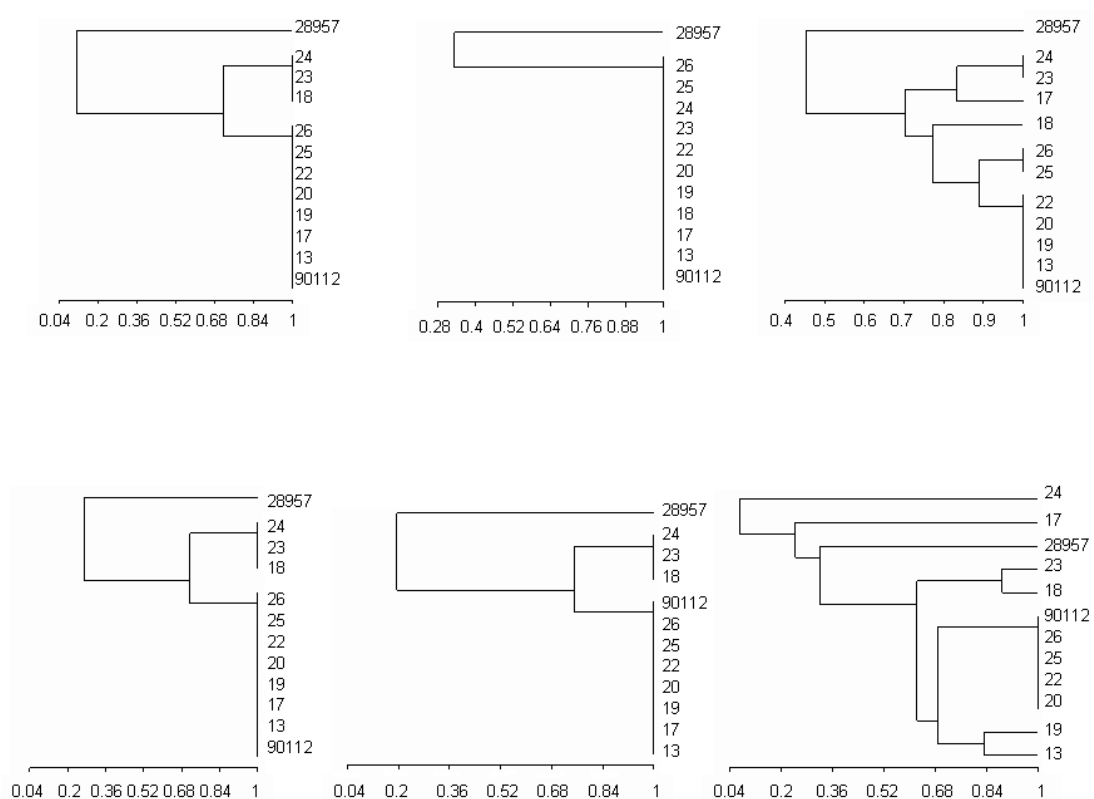
Figure 3. Dendrograms showing the relationships of *C. neoformans* isolates generated from the RAPD-PCR profiles obtained with primers (from left to right) CAV1, CAV2, ZAP19 (above); ZAP20, OPB11 and SEQ6 (below).
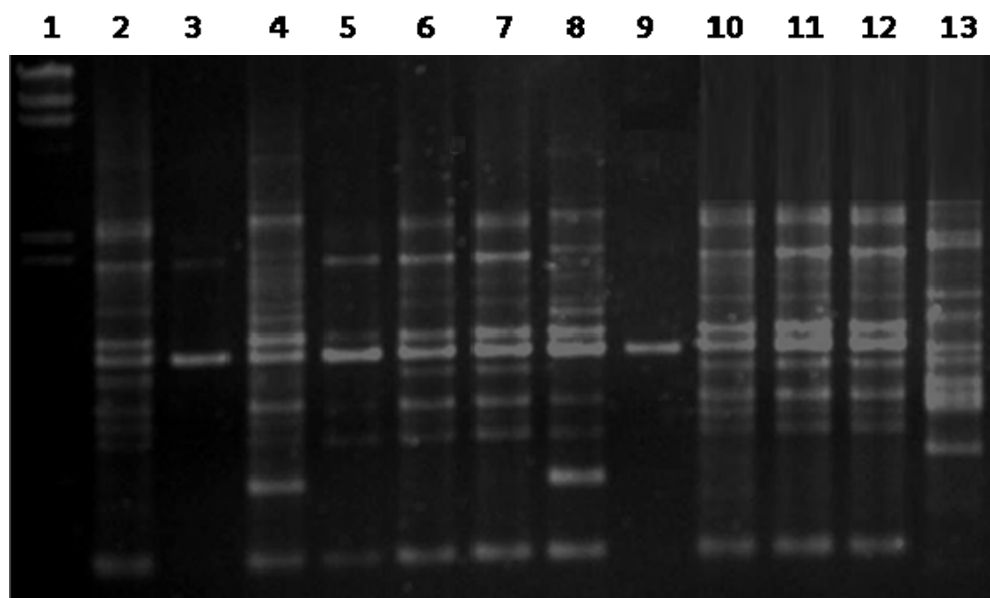

Figure 4. Examples of amplification obtained with the primer SEQ6. Line 1: molecular size marker (Lambda DNA/Hind III; Fermentas); lines 2 to 13 represent *C. neoformans* isolates 13, 17, 18, 19, 20, 22, 23, 24, 25, 26, ATCC 90112 and ATCC 28957, respectively.

DISCUSSION

Molecular techniques have been widely used to study genetic variations in uni- and multicellular organisms (1-4, 28), and RAPD-PCR has been used in studies of human pathogenic fungi as well as those causing diseases in animals and plants (1, 28). Molecular studies of microorganisms contribute to the epidemiological studies and are also useful in the relationship between molecular profile and phenotypic characteristics (12).

In this study, we observed high genetic variability among isolates of *C. laurentii* complex with all the primers used, as demonstrated in previous studies (19, 23, 25). The greatest differences among isolates of *C. laurentii* complex (minor S$_j$) were observed with the primers CAV2, ZAP19, OPB11 and SEQ6. That heterogeneity is probably caused by different species and subspecies that constitute the *C. laurentii* complex, which can only be identified by more specific methods, such as rDNA sequencing (D1/D2 and/or ITS regions).

In clinical or medical laboratory, especially in Brazil, the laboratories are able to identify the *C. laurentii* complex, but not the accurate specie or subspecie. In this line, the use of molecular methods such as RAPD-PCR associated with the phenotypic tests can help identify the species or subspecies of the microorganisms and also their typing.

C. albidus is another specie that is reported as a species complex. In some isolates have been observed a high genetic heterogeneity, revealed by different molecular methods (1, 19, 24). However, in this study we demonstrated that most isolates had high similarity or were identicals (Figure 2). Among the primer used, we observed that CAV2 did not amplify any bands. Despite the small number of *C. albidus* isolates studied, the CAV2 primer seems inappropriate and ZAP20, OPB11 and SEQ6 were the primers that formed more clusters and are most promising for use in studies envolving *C. albidus*.

Isolates of *C. neoformans* were best discriminated by primer SEQ6, that formed eight groups, one of them grouping five isolates, including strain ATCC 90112, a strain that belongs to the var. *grubii* isolated from the cerebrospinal fluid in the United States. The identity revealed by most isolates of *C. neoformans* suggests that new primers should be researched to increase the discriminatory power. Among the primers utilized, we noted that *C. neoformans* ATCC 28957, a strain of the var. *neoformans*, formed an evident separate group with all the primers. It is interesting to note that the primer CAV2 grouped all isolates except 28957, as shown in Figure 3.

The study of molecular markers is promising for the typing of microorganisms. These studies are interesting to study the relationship between molecular profile and phenotypic characteristics, such as *in vitro* virulence factors production (laccases, phospholipases, proteases), phenotypic switching, resistance to antifungal drugs, or associations between geographic origin of the isolates and molecular profiles. Certain molecular profiles may be related to specific features of the phenotype, as has been shown for *C. neoformans* and another species isolates from different parts of the world (1, 3, 4, 10, 14, 26). Recently the evidence of the relationship between susceptibility to antifungal agents and different genotypes of *C. neoformans/ C. gattii* complex has increased (26).

The use of a single molecular marker is usually not sufficient to discriminate a large number of isolates. The establishment of a rational screening protocol, with primers carefully chosen and carried out in sequential reactions, or even the sequential analysis of results can allow discrimination of isolates or even increase the discriminatory power of the method, expanding the field of application of molecular markers. More studies with larger numbers of isolates and primers, available for practical application in clinical and research laboratories, will facilitate the work of researchers, will reduce cost and time consuming.

In conclusion, we observed that the use of limited number of carefully selected primers allowed the discrimination of different isolates, and not every primers (e.g., CAV2 for *C. albidus*) can be applied to any species. Future studies involving

C. laurentii complex will be required to detail the identification at the level of the species or subspecies, for example, by comparison with a validated method such as rDNA sequencing (D1/D2 domain or ITS). More studies, however, are necessary to determine the effectiveness of this tool and primers studied here and other in clinical isolates, as well as in veterinary and environmental isolates.

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