Molecular studies on the rDNA ITS and ISSR regions for identification of *Candida albicans*, *Cryptococcus neoformans* and *Microsporum canis* using universal primers

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Running head
Studies on the rDNA ITS and ISSR regions of *Cr. neoformans, C. albicans and M. canis*

Key words: Molecular identification, *Cryptococcus neoformans*, *Candida albicans*, *Microsporum canis*, inter simple sequence repeats (ISSR), Microsatellites, or simple sequence repeats (SSRs), PCR, ISSR-RAPD

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Abstract:

The traditional molecular markers have, in general, provided insufficient statistical power and accuracy for estimating genetic differences, but the discovery of highly variable loci such as microsatellites means that the statistical power is available for determining differentiation between species groups. Microsatellites, or simple sequence repeats (SSRs), have been the most widely applied class of molecular markers used in genetic studies.

The present studies were focused on the rDNA ITS and ISSR regions of Candida albicans, Cryptococcus neoformans and Microsporum canis, aiming to identify the three fungi to a species level by PCR using universal primers and PCR-restriction fragment length polymorphism (RFLP) techniques. The genomic DNAs of the three fungal species were amplified by PCR using common universal primer sets ([GACA]_{4} and ITS_{1}, ITS_{2},ITS_{3}], followed by RFLPs by two endonucleases enzymes (Bam HI and Hae III). The PCR products generated by ITS primers were digested with restriction enzymes, and the restriction profiles were analyzed.

Interspecies and interstrain genotyping by Inter Simple Sequence Repeat (ISSR-RAPD) fingerprinting was successful. The primer (GACA)_{4} successfully amplified 3 DNA fragments from the reference strain and 8 field isolates Cryptococcus neoformans, recovered from pigeon droppings. The (GACA)_{4}- ISSR RAPD profiles were unique for each fungal species. The primer produced a distinctive and reproducible profile for all Cryptococcus neoformans isolates, where the 3 major bands had average molecular weights of 462, 552 and 827 bp. All Candida albicans isolates and reference strain produced a species specific profile with a similar 5 major band pattern of the average molecular weights 305, 366, 490, 580, and 699 bp In case of Microsporum canis isolates, 6 major bands of average molecular weights of 245, 301, 380, 512, 715 and 924 bp were obtained.

The intergenic spacer region was successfully amplified from all isolates tested and reference control. M. canis was characterized by the largest size of amplicon (756 bp). The problem remained with the PCR products of C. albicans (529bp), and Cr. neoformans (557bp), which were almost similar in size. The RFLP analysis following digestion of the PCR products of these two species by the restriction enzymes Hae III, and Bam HI revealed that the restriction profiles of the Cr. neoformans and C. albicans ITS-amplicons obtained with each enzyme for each isolate were species specific. the restriction enzyme Bam HI digested the amplicon of C. albicans only into two fragments but not in Cr. neoformans, while Hae III enzyme digested the amplicons of C. albicans and C. neoformans into two fragments of different sizes. This was satisfactory for differentiation of both organisms.

We conclude that PCR and PCR-RFLP techniques targeting the rDNA ITS region and ISSR-RAPD profiles are simple, rapid, and quite useful tools for the identification of Candida albicans, Cryptococcus neoformans and Microsporum canis.
1. Introduction:

The molecular identification of fungi has typically concentrated on a single species or genus of fungi so, for molecular diagnosis of fungal diseases using DNA amplification procedures in the routine laboratory, choice of appropriate target structures and rapid and inexpensive identification of amplification products are important prerequisites. The traditional molecular markers have, in general, provided insufficient statistical power and accuracy for estimating genetic differences, but the discovery of highly variable loci such as microsatellites means that the statistical power is available for determining differentiation between species groups\textsuperscript{[1,2]}.

Microsatellites, or simple sequence repeats (SSRs), have been the most widely applied class of molecular markers used in genetic studies, with applications in many fields of genetics including genetic conservation, population genetics, molecular breeding, and paternity testing. This range of applications is due to the fact that microsatellite markers are co-dominant and multi-allelic, are highly reproducible, have high-resolution and are based on the polymerase chain reaction (PCR).

SSRs are non-coding repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem, which are widespread in both eukaryotic and prokaryotic genomes\textsuperscript{[3, 4]}. For that reason, many authors suggested the use of microsatellite as genetic markers to identify individual genetic variability among closely related humans, animals and among plants and fungi\textsuperscript{[5-12]}. 
The aim of the present work was to use ISSR for genotyping of field and standard isolates of *Cr. neoformans* *C. albicans* and *M. canis*, commonly isolated in Egypt, by the universal primer (GACA)_4

2. Materials and Methods

2.1. Standard and field isolates:

The standard strain, *Cr. neoformans* serotype A (ATCC 90112 CSF Pennsylvania isolate) was obtained from the American Type Culture Collection, while, the *C. albicans* *M. canis* and *Cr. neoformans* isolates were obtained from the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

2.2. Media :

Sabouraud dextrose agar (SDA) medium with chloramphenicol: and Sabouraud broth (SB) (Difco Lab., Detroit, MI, USA) were used for preparation of yeast isolates for DNA extraction.

2.3. Primers: (Promega Corp., Madison, Wis)

A) Internal Transcribed regions:
ITS1 (5′-TCCGTAGGTGAACCTGCG-3′),
ITS3 (5′GCATCGATGAAGAAGCAGC-3′),
ITS4 (5′-TCCCTCCGCTATTG ATATGC-3′).

B) Nonanchored ISSR primer:
Universal microsatellite DNA primer (GACA)_4 is a single oligonucleotide complementary to single repetitive sequences present in the target DNA. This repetitive sequence is called microsatellites.

2.4. Buffers and reagents (Promega Corp., Madison, Wis)

Fungal lysis buffer, Potassium acetate buffer (pH 4.8), PCR Master Mix, DNA Ladder Markers, endonuclease enzymes (*Bam HI* and *Hae III*), Tris-borate EDTA buffer, Ethidium bromide, gel loading buffer.[13,14]

2.5. Equipment and Soft wares:

GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.), Gel analyzer 3 EGYGENE, SPSS 11.5 statistical analysis

2.6. Rapid mini–preparation DNA Extraction for all fungal isolates:
It is the technique of Liu et al. (2002)\textsuperscript{(13)} for dermatophytes fungi but with a slight modification to fit for the three fungal species in our study. To a 10 ml tube containing 5 ml of lysis buffer (400mM Tris-HCl, pH 8.0, 60 Mm EDTA, pH 8.0, 150 Mm NaCl, SDS 1% w/v), a large portion of *M. canis* mycelium grown on SDA in primary culture was added using a sterile tooth pick, with which the mycelia were disrupted with a glass homogenizer (in case of yeast more than colonies were collected). The tube was then left at room temperature for 10 min. after adding 1.5 ml of potassium acetate, pH 4.8 (5 M potassium acetate 60ml glacial acetic acid 11.5 ml, distilled water 28.5 ml), the tube was incubated at 90°C for 1 hr. then, vortex mixed briefly, and cellular debris and precipitated protein were removed by centrifugation at > 13000 rpm for 1 min. The supernatant was transferred to another tube and centrifuged again as above. After transferring the supernatant to a new tube, an equal volume of cold isopropanol alcohol was added then, incubated at -20°C/ 1 hr or -80°C / 10 min. The tube was mixed briefly by inversion, centrifuged at > 13000 rpm for 2 min and the supernatant was discarded. The resultant DNA pellet was washed in sufficient amount of ethanol 70% v/v. after centrifugation at 13000 rpm for 1 min, the supernatant was discarded. The DNA pellet was air dried and dissolved in 100 µl of 1X TE; 1 µl of the purified DNA was used in 25-50 µl of PCR mixture. The same technique was applied to culture of *Cr. neoformans* and *C. albicans and M. canis*

2.6. Amplification of universal Inter Simple Sequence Repeat (ISSR-RAPD) for all fungal isolates\textsuperscript{[10, 15, 16]}:

PCR amplification reactions were performed with volumes of 50 µl containing 10 to 25 ng of genomic DNA of various isolates of *Cr. neoformans* and *C. albicans* and *M. canis*, 20 to 30 ng of primer (GACA)\textsubscript{8}. Under the recommended buffer conditions. The PCR was performed as follows: initial denaturation at 94°C for 4 min, then 25-30 cycles of denaturation at 94°C for 30 Sec., annealing at 55°C for 30 Sec. and extension 72°C for 1 min and final extension for 4 min. Amplification products (20 µl / sample) were separated by electrophoresis in 2 % agarose gels for 90 min in 0.5x TBE buffer. Amplification products were detected by staining with ethidium bromide and were visualized under UV trans-illuminator and photographed by a digital camera (FUJI 100). PCR fingerprinting profiles were sized and compared with a scanner and gel image analysis software

7. Amplification of ITS Region of rDNA fragment in all isolates by a universal two sets of ITS-PCR primers\textsuperscript{[10, 15, 16]}:

ITS1, ITS3, and ITS4 primers targeting the conserved regions of 18S, 5.8S, and 28S rDNAs, respectively, were used for amplification. The ITS1-ITS4 primer pair was used to amplify the intervening 5.8S rDNA, the adjacent ITS1 region and ITS2 region, but the ITS3-ITS4 primer pair was used to amplify a portion of the 5.8S rDNA and the adjacent ITS2 region.

The PCR amplification reaction was performed with a volume of 50 µl. Twenty five µl of PCR Master Mix (2X), Consisted of 1-2 µl (25ng-50ng DNA template) of each sample was added to the PCR master mixture, 0.5 µl of each primer (10 pmol of each primer), and the remaining volume consisting of nuclease free distilled water. The PCR mixture after each step, was mixed thoroughly with the vortex and kept on ice. The PCR mixture was transferred to PCR tubes in aliquots. The PCR reaction mixture was overlaid with 75µl mineral oil to prevent evaporation.

Amplification consisted of an initial denaturation at 94°C for 4 min, then 30
cycles of denaturation at 94°C for 30 min, annealing at 55°C for 30 min and extension 72°C for 1 and 4 min. Gene Amp PCR system 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) was used. Negative control reactions without any template DNA were carried out simultaneously. Gel electrophoresis with 1.5% agarose gels was conducted with 1xTBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 4.8 V/cm for 2 h. A 100-bp DNA ladder (Promega Corp., Madison, Wis.) was run concurrently with amplicons for sizing of the bands. Gels were stained with ethidium bromide-TBE solution for 20 min and the obtained bands were visualized using UV-trans-illuminator and photographed by a digital camera (FUJI 100). Accurate fragment size analysis based on the electrophoretic mobility of the sample relative to the internal standards (100 bp ladder promega) was achieved by using DNA size analysis software.

2.8. Restriction digest of ITS1-ITS4 Primers amplicons (PCR-RFLP) [17]

This technique had been successfully applied using the ribosomal DNA region including the intergenic spacers ITS1 and ITS2, and the 5.8S rRNA encoding gene. Total purified PCR product (Amplicon) Cr. neoformans (540 – 569bp) and C. albicans (527bp) was digested individually with 20 U of the restriction enzymes Hae III or Bam HI, by 1hr – 16 hr incubation at 37°C in reaction mixture containing 10 µl of PCR product, 18 µl of water, 2.0 µl of 10X Buffer R. The resulting restriction fragments were analyzed by 2% agarose gel electrophoresis and DNA ladder of 1kb was used. Obtained bands were visualized using UV-trans-illuminator and photographed by a digital camera (FUJI 100). Accurate fragment size analysis based on the electrophoretic mobility and was determined by using DNA size GEL ANALYZER 3 EGYGENE analysis software.

3. Results

3.1. Interspecies and interstrain genotyping by Inter Simple Sequence Repeat (ISSR-RAPD) fingerprinting:

The primer (GACA)_4 successfully amplified 3 DNA fragments from the reference strain and 8 field isolates of Cryptococcus neoformans, recovered from pigeon droppings. The primer produced a distinctive and reproducible profile for all Cryptococcus neoformans isolates, where the 3 major bands had average molecular weights of 462, 552 and 827 bp (Table 1 and Photo 1).

Table 1: Average molecular sizes of major diagnostic bands of Cr Cryptococcus neoformans Candida albicans and Microsporum canis obtained with the microsatellite primer (GACA)_4

<table>
<thead>
<tr>
<th>Bands</th>
<th>Cryptococcus neoformans</th>
<th>Candida albicans</th>
<th>Microsporum canis</th>
</tr>
</thead>
</table>
the average molecular weight produced a species specific profile with a similar reference strain, when tested by the microsatellite primer (GACA) as shown in (1:G, A, C, U, S, R, A, G, C).

As shown in Table 1 and Photo 2, all Candida albicans isolates and reference strain, when tested by the microsatellite primer (GACA)$_4$, produced a species specific profile with a similar 5 major band pattern of the average molecular weight 305, 366, 490, 580, and 699 bp.
In case of *Microsporum canis* field and reference isolates, 6 major bands of average molecular weights of 245, 301, 380, 512, 715 and 924 bp were obtained (Table 1 and Photo 3).

![Photo 3: ISSR-RAPD fingerprints of reference strain and different isolates of *M. canis* species after electrophoretical separation generated by single primer (GACA)n, (Marker) (200) bp DNA Ladder Marker (Jena Bioscience) (Negative) water (blank).](image)

### 3.2. ISSR polymorphism percent:

The banding patterns, obtained by GEL ANALYZER3, revealed the number of ISSR fragments produced from each species either for the reference strain or the isolates, according to their frequency and the polymorphism depending on (0 – 1) percent, as absence (0.000) or presence (1.000). The data indicated that all three species produced 8 polymorphic bands and only one common monomorphic band, where *Cr. neoformans* produced 2, *C. albicans* 4 and *M. canis* 5 bands. All the eight ISSR markers were polymorphic among the 3 strains and the polymorphism percent was 88.89 between three fungal species (calculated with analysis software according to Nei's analysis)\(^\text{29}\).
The similarity coefficient was determined both within and between groups (species). The data obtained from the analysis of ISSR banding patterns of three fungal species (Binary matrix) were analyzed by SPSS version-11.5 program to obtain similarity matrix. The similarity coefficient values among the studied three fungal species were calculated according to Dice's coefficient \[^{[30]}\]. The results indicated an average genetic distance ranging from 0.222 to 0.727. The highest similarity index (0.727) was recorded between \textit{C. albicans} and \textit{M. canis}. However, the lowest similarity index (0.222) was observed between \textit{Cr. neoformans} and \textit{M. canis}.

The similarity matrix data was used to draw precise relationships among the three tested fungal species. The dendogram was constructed from the binary matrix data of 9 ISSR markers. As shown in Fig. 1, two main clusters were obtained. The first cluster corresponded to reference strain and isolates of \textit{C. albicans} and \textit{M. canis}. The second cluster constituted only reference strain and isolates of \textit{Cr. neoformans}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{dendrogram.png}
\caption{Dendrogram and cluster analysis of \textit{Cr. neoformans}, \textit{C. albicans} and \textit{M. canis} similarity based on analyzing of ISSR fingerprints data produced by microsatellite DNA primer \textit{(GACA)}\textsubscript{4}, based on Dice's similarity and UPGMA method.}
\end{figure}
3.4. The use of primers ITS3-ITS4 as a universal primer pairs

The use of ITS3-ITS4 as a universal primer pairs in order to detect the three species in one PCR step revealed that the obtained PCR amplicons were of almost the same average ranges (Photo 4 and Table 2)

![Photo 4: Electrophoretic profile of PCR products of C. albicans, Cr. neoformans and M. canis isolates amplified by using the primer (ITS3 -ITS4). (Marker) 100bp., (R.)reference strain, (I.) isolate](image)

**Table 2: Results of C. albicans, Cr. neoformans and M. canis positive PCR products amplified by using the primer (ITS3-ITS4) by Gel analyzer 3 program.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Candida albicans</th>
<th>Cryptococcus neoformans</th>
<th>Microsporum canis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Mol. Wt.</td>
<td>328</td>
<td>339</td>
<td>363</td>
</tr>
</tbody>
</table>
The use of the primers ITS1-ITS4 revealed that *C. albicans* produced a PCR product of (529bp), *Cr. neoformans* PCR products was slightly larger (557bp) while, *M. canis* PCR amplicon was the largest (765 bp) (Photo 5 and Table 3).

![Electrophoretic profile of PCR products of C. albicans, Cr. neoformans and M. canis amplified by using the primer (ITS1 -ITS4). (Marker) 100bp, (R)reference strain, (I) isolate.](image)

**Photo 5:** Electrophoretic profile of PCR products of *C. albicans*, *Cr. neoformans* and *M. canis* amplified by using the primer (ITS1 -ITS4). (Marker) 100bp., (R)reference strain, (I) isolate.

**Table 3:** Results of *C. albicans*, *Cr. neoformans* and *M. canis* positive PCR products amplified by using the primer (ITS1-ITS4) by Gel analyzer 3 program.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Candida albicans</th>
<th>Cryptococcus neoformans</th>
<th>Microsporum canis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>R</em></td>
<td><em>I</em></td>
<td><em>R</em></td>
</tr>
<tr>
<td>Mol. Wt.</td>
<td>529</td>
<td>529</td>
<td>551</td>
</tr>
<tr>
<td>AMW</td>
<td>529</td>
<td>557</td>
<td></td>
</tr>
</tbody>
</table>

3.5. Restriction Fragment Length Polymorphism analysis of PCR product of ITS1-ITS4 Primers of *C. albicans* and *Cr. neoformans* (PCR-RFLP):

As mentioned above, the intergenic spacer region was successfully amplified from all isolates tested and reference control. *M. canis* was characterized by the largest size of amplicon (756 bp), which was diagnostic. The problem remained with the PCR products of *C. albicans*, and *Cr. neoformans*, which were almost similar in size. The RFLP analysis following digestion of the PCR products of these two species by the restriction enzymes *Hae III*, and *Bam HI* revealed specific profiles for each of *Cr. neoformans* and *C. albicans* ITS-amplicons. As shown in Tables 4 and 5 and Photos 6 and 7, the restriction enzyme *Bam HI* digested the amplicon of *C. albicans* only into two fragments, while *Hae III* enzyme digested the amplicons of both *C. albicans* and *Cr. neoformans* into two fragments of different sizes. This was satisfactory for differentiation of both organisms.

![RFLP patterns of PCR products](image)

**Foto 6:** RFLP patterns of PCR products (of ITS1-ITS4 primer) of *C. albicans*, *Cr. neoformans* and *M. canis* after digestion with endonuclease *Bam HI* and electrophoretical separation on a 2% agarose gel. *C. albicans* give a distinct pattern (Marker) 100bp. (R.) reference strain (I.) isolate.

**Table 4:** Results of RFLP patterns of *C. albicans*, *Cr. neoformans* and (ITS1-ITS4) PCR products after digestion by *Bam HI* enzyme.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Candida albicans</th>
<th>Cryptococcus neoformans</th>
<th>Microsporum canis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Bam HI digest fragments</td>
<td>296</td>
<td>296</td>
<td>537</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>228</td>
<td>-----</td>
</tr>
</tbody>
</table>

(R.): Reference strains, (I.): isolates, C.: C. albicans, Cr. n.: Cr. neoformans,

**Foto 7:** RFLP patterns of *C. albicans* and *Cr. neoformans* ITS1-ITS4 PCR products after digestion with endonuclease

**Table 5:** Results of RFLP patterns of *C. albicans*, and *Cr. neoformans* (ITS1-ITS4) PCR products after digestion by *Hae III* enzyme.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Candida albicans</th>
<th>Cryptococcus neoformans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R.)</td>
<td>(I.)</td>
</tr>
<tr>
<td>Hae III digest fragments</td>
<td>365</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>117</td>
</tr>
</tbody>
</table>

(R.): Reference strains, (I.): isolates, Cr. neo: Cr. neoformans,
Discussion

ISSR is a semi arbitrary molecular technique involving the use of one primer complementary to a target microsatellite region in PCR \cite{10, 18, 19, 20}. Amplification in the presence of non-anchored primers also has been called microsatellite-primed PCR (MSP-PCR) \cite{16, 21, 22} or random amplified microsatellite (RAMS) technique \cite{19, 20}.

The primer (GACA)$_4$ is the most common primer used in the amplification of ISSR regions as it was used on *Cr. neoformans* and closely related species \cite{10}, on *Cr. neoformans* var. grubii and var. *neoformans* \cite{17, 23}, on *Cr. neoformans* var. gattii and var. *neoformans* \cite{24} and on genotyping of *Cr. neoformans* var. *neoformans* \cite{25}.

In the present work, ISSR was used for genotyping of all isolates of the three fungi. The universal primer (GACA)$_4$ produced a distinctive and reproducible profile for all *Cr. neoformans* isolates with three major bands of average size of 810, 552 and 463. According to Cogliati et al. (2007) \cite{25}, the pattern of bands obtained in the present study is diagnostic for *Cr. neoformans* var. *grubii*.

All *Candida* isolates tested with microsatellite primer produced 5 major bands of average molecular weight of 305, 366, 490, 580 and 699 bp. These results conform to that obtained by other authors \cite{15, 21, 26, 27}.

Similarly, all *M. canis* isolates tested by this primer produced a species specific profile with 6 major bands, which are diagnostic for this dermatophyte. This result confirms the findings of other authors \cite{15, 16, 28}.

It can be concluded, that the single ISSR primer (GACA)$_4$ was successfully used as a genetic marker for three fungal species. It is quite clear, that the ISSR single primer (GACA)$_4$ is of utmost importance for *Cr. neoformans* typing. It is of interest that all tested isolates could be
typed as *Cr. neoformans* var. *grubii* according to *Viviani et al.* (1997) \[23\] and *Cogliati et al.* (2007) \[25\].

A total of 9 bands were amplified by the ISSR in the three fungal species, 2 bands for *Cr. neoformans* var. *grubii*, 4 bands for *C. albicans* and 5 bands for *M. canis*. Only one band was monomorphic and the other eight bands were polymorphic. By analyzing the ISSR banding patterns of (GACA)\(_4\), the polymorphism percent among the three fungal species was 88.89 \[29\], while similarity coefficient ranged between 0.2 to 0.7. \[30\]. The statistical analysis of polymorphism and similarity coefficient, as calculated by Un weighted Pair-Group Method using arithmetic Averages (UPGMA) for the tested isolates and reference strains of three fungal species revealed that the highest similarity was between *C. albicans* and *M. canis*, where both were clustered in one group, while the lowest similarity was between *Cr. neoformans* and *M. canis*.

Many authors referred to ITS regions as a pan fungal molecular identification tool; however, the approaches of depending on that operon were divided into two groups. The first group succeeded in identification of different fungal species depending on polymorphism in sequence analysis of that region on *Candida* species \[31\]; on dermatophyte species \[32,33\]; on *Cr. neoformans* \[34\] and on *M. canis* \[35,36 and 37\].

On the other hand, the second group of authors achieved accurate fungal identification depending on length polymorphism in the ITS and the adjacent regions. They mostly depended on length variation of ITS 2 regions only, while others identified fungal species by both regions, ITS1 and ITS2 in combination, e.g. on the ITS 1, 5.8S, and ITS 2 region \[38\]; on differences in the size of the ITS2 regions only \[39\]; on ITS1- 5.8S rDNA-ITS2 regions \[40\]; on ITS1 and ITS2 \[41\]; on ITS2 only \[42\].

In the present study, the ITS regions of rDNA were chosen to be the target for pan molecular identification of *Cr. neoformans*, *C. albicans*
and *M. canis* with a universal (ITS) fungus-specific set of primers. The intergenic spacer regions were successfully amplified from all tested isolates and reference control. The ITS3-ITS4 primers (for ITS2 and a part of 5.8S rDNA) amplified PCR products of the following average sizes: *M. canis* (319 bp), *C. albicans* (343bp) and *Cr. neoformans* (363bp) such ranges do not allow precise differentiation between the three species. Thus, it could be concluded, that these regions alone don't represent a universal diagnostic tool for the three fungal species of that study.

Accordingly, all the isolates and reference control were tested by the other primers pair ITS1-ITS4 (for amplification of ITS1, ITS2 and 5.8S rDNA), where all *M. canis* isolates produced a unique PCR product of approximately (725 bp), while isolates of *Cr. neoformans* yielded products of (556bp) and *C. albicans* (529 bp).

It is worthy to mention, that the ITS1-ITS4 primers PCR products of *C. albicans* and *Cr. neoformans* contained unique ITS alleles which were distinguishable by restriction enzyme analysis. Some authors regarded the 5.8S gene to have a highly conserved sequence but with a low intraspecific variability, which is not enough to delimitate between co specific strains. However, the ITS regions, which are non-coding hyper variable ones, could permit the identification at the intra or interspecific levels. The hyper variability of sequence analysis of the three regions together was reported\(^{32, 34, 44, and 45}\).

The use of PCR-RFLP as a rapid molecular identification technique for many fungal strains was recorded, e.g. *Candida krusei*\(^{46, 47}\), *Aspergillus fumigatus* \(^{48}\); *C. albicans* and *Cr. neoformans*\(^{35, 51, 52}\); *T. mentagrophytes* var. *interdigitale* \(^{50}\); *Candida* species\(^{49, 40, 41}\) and this in turn substantiate our findings, provided that the proper primers and restriction enzymes are used.
These findings encourage the testing of more fungi by these primers and determine the possibility of application of the technique directly on clinical samples.

References


Cruz, Rio de Janeiro, 99(2): 147-152.
Photo 4: ISSR-RAPD fingerprints of reference strain and isolates of *Cr. neoformans*, *C. albicans* and *M. canis* species after electrophoretical separation generated by single primer (GACA)$_4$, (Marker) (200) bp DNA Ladder Marker.