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Genetic characterization of Brazilian strains of *Aspergillus flavus* using DNA markers

P.P. Batista[1], J.F. Santos[1], N.T. Oliveira[2], A.P.D. Pires[2], C.M.S. Motta[2] and E.A. Luna-Alves Lima[2]

[1]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Departamento de Micologia, Universidade Federal de Pernambuco, Recife, PE, Brasil

Corresponding author: E.A. Luna-Alves Lima
E-mail: ealal@bol.com.br

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ABSTRACT. The *Aspergillus* genus belongs to a filamentous fungal group characterized by wide dispersion in the environment. Some species are associated with diseases, especially in immunocompromised patients, while others are of economical importance due to aflatoxin production or biotechnological applications. Its species identification is nowadays performed by traditional techniques combined with molecular markers, resulting in a higher efficiency of isolate characterization. In the present study, internal transcribed spacer, inter-simple sequence repeats (ISSR), and random amplified polymorphic DNA (RAPD) molecular markers were used, with the aim of genetically characterizing strains of *Aspergillus flavus* and strains of other species of the *A. flavus* group. High genetic diversity was revealed by RAPD and by ISSR, in which the use of the $(GACA)_4$ primer yielded a higher diversity than with the $(GTG)_5$ primer, although the latter showed a characteristic banding profile for each species. These data were used to create a similarity matrix for the construction of dendrograms by means of the UPGMA method. The ISSR and RAPD profiles showed that among the strains previously identificated as *A. flavus*, one should be *A. oryzae*, one *A. parasiticus* and two *A. tamarii*. On the other hand, a strain previously identified as *A. parasiticus* should be *A. flavus*. All these strains

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were retested by traditional methods and their new species identification was confirmed. These results strongly support the need for using molecular markers as an auxiliary tool in differentiating fungal species and strains.

Key words: *Aspergillus flavus*; Genetic diversity; Molecular markers; Random amplified polymorphic DNA; Internal transcribed spacer; Inter-simple sequence repeats

INTRODUCTION

The genus *Aspergillus* belongs to a filamentous fungal group with a wide dispersion in the environment and consists of approximately 200 species (Raper and Fennell, 1977). Several *Aspergillus* species have received more attention due to biotechnological applications as well as public health problems. Some *Aspergillus* species are of medical significance, since they are opportunistic pathogens in humans (Verweij et al., 1996; Diaz-Guerra et al., 2000; Bertout et al., 2001). The genus can be divided into two main groups of species. The first group includes *A. flavus*, *A. parasiticus* and *A. nomius*, species that are aflatoxin producers, and the second group includes *A. oryzae*, *A. sojae* and *A. tamarii*, which are used in the fermenting process in Asian countries (Kumeda and Asao, 2001). However, there are suggestions that *A. oryzae* and *A. sojae* are in fact domesticated strains of *A. flavus* and *A. parasiticus*, respectively (Kurtzman et al., 1986).

Traditional methods for species identification are mainly based on morphological parameters, including colony diameter, color and texture, size and texture of conidia and conidiophore structure (Klick, 2002). However, species classification may be difficult due to extensive divergence of morphological characters produced by a high level of genetic variability both inter- and intraspecific (Kumeda and Asao, 1996). Despite intense investigation, the taxonomy of this group of fungi is still highly complex and the genetic techniques to detect the level of polymorphism and similarity among species help minimize the problem. These techniques involve random amplified polymorphic DNA (RAPD) markers, restriction fragment length polymorphism, amplified fragment length polymorphism, inter-simple sequence repeats (ISSR), internal transcribed spacer (ITS), intron, and single-strand conformation polymorphism, which are used as auxiliary tools in traditional methods (Kumeda and Asao, 2001; Dendis et al., 2003). The high degree of genetic variations of nucleotide sequences of the ITS1-5.8S-ITS2 region makes the comparison among *Aspergillus* species very useful for strain classification and phylogenetic studies (Kumeda and Asao, 1996; Henry et al., 2000). Species identification using ITS markers can be even more reliable than large ribosomal subunit D1-D2 domain sequence analysis (Hinrikson et al., 2005). RAPD markers also significantly contribute to the classification of *Aspergillus* species (Rath, 2001).

The objective of the present study was to genetically characterize several strains of the *Aspergillus flavus* group isolated from different sources and deposited at the certified URM Micoteca (Recife, Brazil), using polymerase chain reaction (PCR) amplification of molecular markers. This is the first demonstration of using ISSR markers to characterize *Aspergillus* species. Unlike ITS, the RAPD and ISSR profiles were of substantial importance as auxiliary tools for the reclassification of strains using traditional methods.

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MATERIAL AND METHODS

Fungal strains

The fungal strains used in this study were obtained from the Fungi Culture Collection of the certified Micoteca URM of Departamento de Micologia of Universidade Federal de Pernambuco (Recife, PE, Brazil). A list of the 25 strains of the *Aspergillus flavus* group studied, along with 5 strains previously classified as *A. flavus* by traditional methods, including 2 strains of *A. parasiticus*, 2 strains of *A. oryzae* and 1 strain of *A. sojae*, for comparison, are presented in Table 1. A strain of *A. niger* was also included as outgroup for dendrogram construction. All strains were maintained in PDA (Oxoid) medium at 4°C.

Table 1. Strains of the *Aspergillus flavus* species group used in the present study, identified by accession numbers in the internationally certified Micoteca URM (Departamento de Micologia, Universidade Federal de Pernambuco, PE, Brazil).

Strain	URM accession number	Species	Substrate or host	Geographical origin	Year registered
1	036	*Aspergillus flavus*	-	Pernambuco	1954
2	221	*Aspergillus flavus*	-	Pernambuco	1957
3	1869	*Aspergillus flavus*	Soil	Amapá	1969
4	2226	*Aspergillus flavus*	Bronchial wash	Pernambuco	1968
5	2487	*Aspergillus flavus*	Ground foliage	Pernambuco	1978
6	2578	*Aspergillus flavus*	Soybean seeds	Rio Grande do Sul	1980
7	2701	*Aspergillus flavus*	Contaminant	Brasília	1981
8	2814	*Aspergillus flavus*	Sugar cane remnants	Alagoas	1985
9	3091	*Aspergillus flavus*	*Rudigea gardenoides*	São Paulo	1989
10	3426	*Aspergillus flavus*	Oats	Pernambuco	1994
11	3739	*Aspergillus flavus*	Water	Pernambuco	1997
12	4365	*Aspergillus flavus*	Soil	Pernambuco	2001
13	4509	*Aspergillus flavus*	Soil	Pernambuco	2002
14	4526	*Aspergillus flavus*	Maize powder	Pernambuco	2002
15	4541	*Aspergillus flavus*	Maize powder	Pernambuco	2002
16	4709	*Aspergillus flavus*	Soil	Pernambuco	2003
17	4928	*Aspergillus flavus*	Soil	Pernambuco	2005
18	4933	*Aspergillus parasiticus*	Soil	Pernambuco	2005
19	4999	*Aspergillus flavus*	Tea	Pernambuco	2005
20	5167	*Aspergillus flavus*	Human skin	Rio de Janeiro	2005
21	648	*Aspergillus flavus*	Ear secretion	Pernambuco	1956
22	433	*Aspergillus oryzae*	Air	Bahia	1955
23	951	*Aspergillus sojae*	-	Japan	1957
24	1873	*Aspergillus oryzae*	Soil	Amapá	1873
25	5119	*Aspergillus parasiticus*	Soil	Pernambuco	2005
26	5162	*Aspergillus niger*	Mortar	Pernambuco	2005

Mycelial growth and DNA extraction

Conidia from each strain were suspended in 3 mL 0.1 (v/v) Tween 80 solution and transferred to Ehrlenmeyer flasks with 100 mL Czapeck liquid medium. After inoculation, the flasks were maintained at 250 rpm under agitation at room temperature (±28°C) for 96 h to obtain mycelial growth. Mycelia were collected by vacuum filtration, washed with distilled water and stored at -20°C. DNA extraction was performed according to Raeder and Broda

(1985), resuspended in TE buffer and kept at -20°C until use. All PCR amplifications were performed in a Techne TC-512 thermocycler (Analitica).

ITS region of rDNA amplification

The PCR mixtures were made to a final volume of 50 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 12.5 mM of each primer, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAA-3'), 1.25 U *Taq* DNA polymerase (Invitrogen Life Technologies) and 50 ng genomic DNA (White et al., 1990). Amplification consisted of an initial denaturation step at 95°C for 4 min followed by 40 cycles of denaturation at 92°C for 1 min, annealing at 51.3°C for 1 min and amplification at 72°C for 2 min, with a final extension step at 72°C for 5 min. Amplification products for the ITS locus were separated on a 0.8% agarose gel by electrophoresis at 3 V cm^{-1} in 0.5X TBE buffer (Tris-borate EDTA, pH 8.0), using a 100-bp ladder DNA marker (Invitrogen). The gel was incubated with ethidium bromide and visualized and photographed under UV light.

RAPD by PCR

In order to identify the best primers to establish the RAPD profile, we tested 29 arbitrary oligonucleotides from the OPW, OPA and OPX Kits (Invitrogen), using total DNA from the URM2578 *A. flavus* strain as template. The 5 primers that gave high band number and best reproducibility, OPA-10 (5'-GTGATCGCAG-3'), OPA-14 (5'-TCTGTGCTGG-3'), OPA-17 (5'-GACCGCTTGT-3'), OPA-20 (5'-GTTGCGATCC-3'), OPW-04 (5'-CAGAAGCGGA-3') and OPW-05 (5'-GGCGGATAAG-3'), were chosen to perform the complete RAPD profile analysis of all strains. The PCR mixtures were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTP, 0.4 mM of each primer, 2 U *Taq* DNA polymerase (Invitrogen Life Technologies) and 25 ng genomic DNA (Williams et al., 1990). Amplification consisted of an initial denaturation step at 92°C for 5 min followed by 40 cycles of denaturation at 92°C for 1 min, annealing at 39°C for 1.5 min and amplification at 72°C for 2 min, with a final extension at 72°C for 5 min. Amplification products of RAPD were separated on a 1.4% agarose gel by electrophoresis at 3 V cm^{-1} in 0.5X TBE buffer (Tris-borate EDTA, pH 8.0), using a 500-bp ladder DNA marker (Invitrogen). The gel was incubated with ethidium bromide and visualized and photographed under UV light.

ISSR by PCR

PCR amplification of ISSR was performed with $(GTG)_5$ and $(GACA)_4$ primers. The reaction mixtures were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.25 mM of the primer, 1.25 U *Taq* DNA polymerase (Invitrogen) and 50 ng genomic DNA (Williams et al., 1990). Amplification consisted of an initial denaturation step at 93°C for 5 min followed by 40 cycles of denaturation at 93°C for 20 s, annealing at 55°C for 45 s and amplification at 72°C for 90 s, with a final extension at 72°C for 6 min. The products were separated on 1.4% agarose gel by electrophoresis at 3 V cm^{-1} in 0.5X TBE buffer (Tris-borate EDTA, pH 8.0), using a 100-bp

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ladder DNA marker (Invitrogen). The gel was incubated with ethidium bromide and visualized and photographed under UV light.

RESULTS

ITS amplification products

Using the ITS4 and ITS5 primers, a unique band of about 600 bp was obtained for all strains of *Aspergillus* tested (Figure 1), including the *A. niger* intended to be used as the outgroup for dendrograms.

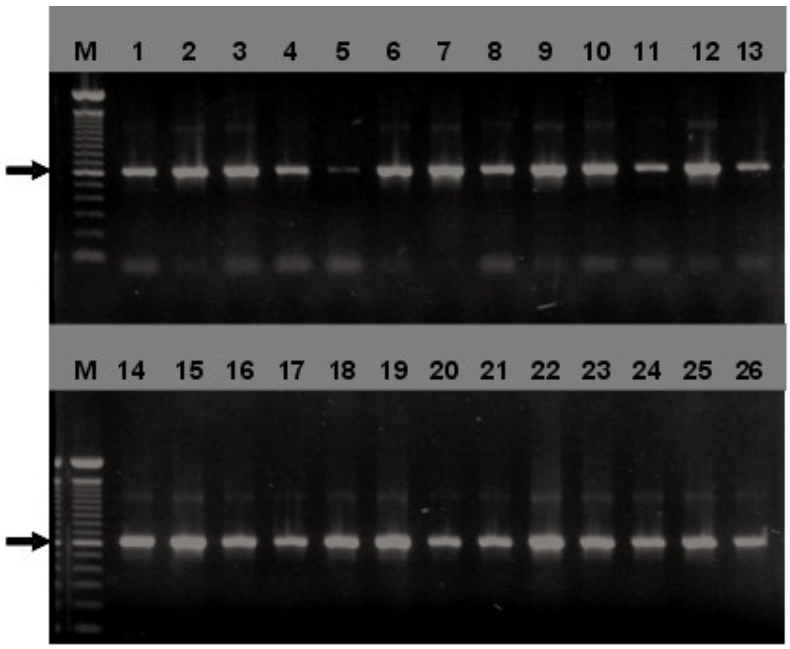

Figure 1. PCR amplification profile of rDNA ITS region of several strains of *Aspergillus flavus* species group (lanes 1 to 25) and *Aspergillus niger* (lane 26). Arrows point to a 600-bp band of a 100-bp DNA ladder marker (M).

ISSR amplification

The amplification profile of ISSR using the $(GTG)_5$ primer showed an average of 12 bands for each of the 20 strains of *A. flavus*, with size variation between 0.4 and 2.0 kb (Figure 2). The similarity in banding patterns shared by the majority of strains agreed with their identification as *A. flavus* by classical methods. However, the strain URM2814 previously classified as *A. flavus* showed a banding pattern similar to that of *A. parasiticus*, and a new evaluation by conventional techniques confirmed its new identification. On the other hand, the

strain URM4933 of *A. parasiticus* displayed a banding pattern similar to that of the *A. flavus* strains, and reexamination by classical methods confirmed it as *A. flavus*. The banding patterns also indicated that the strains URM3091 and URM648 of *A. flavus* should be *A. tamarii*, and similarly the strain URM4709 of *A. flavus* should be *A. oryzae*. These new identifications were confirmed by reexamination using classical methods.

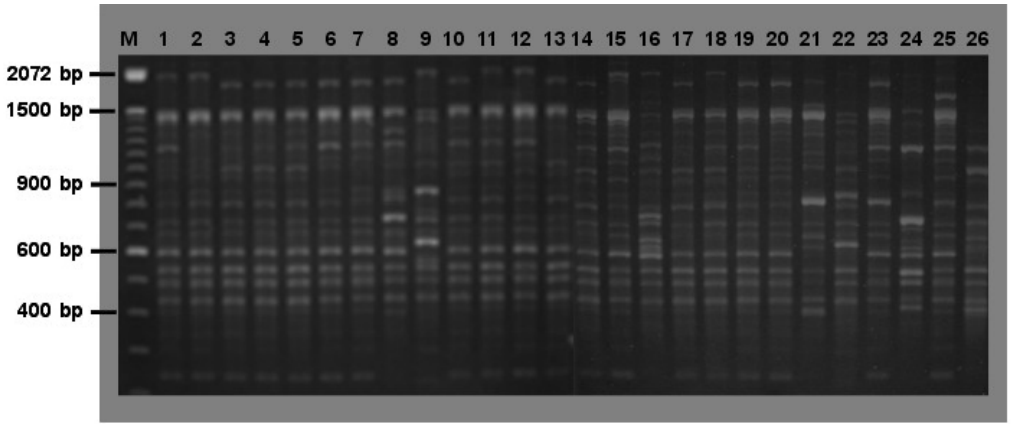

Figure 2. Profile of ISSR amplification by PCR with $(GTG)_5$ primer of several strains of *Aspergillus flavus* species group (lanes 1 to 25) and *Aspergillus niger* (lane 26). M = 100-bp DNA marker.

The ISSR amplification using the $(GACA)_4$ primer produced a variable number of bands in the strains tested, ranging between 0.4 and 2.1 kb (Figure 3). The variation in banding obtained did not evidence a characteristic profile for *A. flavus*.

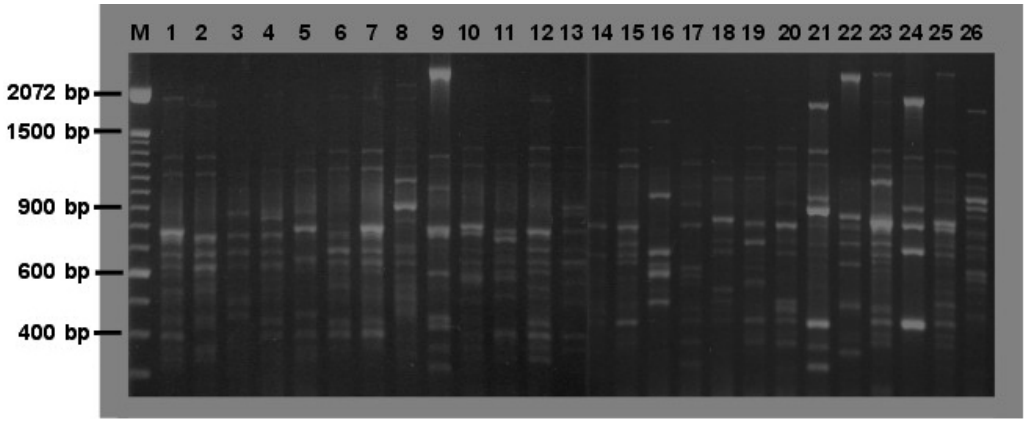

Figure 3. Profile of ISSR amplification by PCR with $(GACA)_4$ primer of several strains of *Aspergillus flavus* species group (lanes 1 to 25) and *Aspergillus niger* (lane 26). M = 100-bp DNA marker.

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Phenetic analysis

The dendrogram generated using the similarity matrix produced with the banding patterns obtained with primers $(GTG)_5$ and $(GACA)_4$ showed the formation of a main group consisting exclusively of *A. flavus* strains, with a similarity level above 80%, although it was divided into two subgroups, indicating considerable internal variation (Figure 4). The first subgroup included the *A. flavus* strain URM4933 previously misidentified as *A. parasiticus*. However, the strains URM2814 and URM5167 did not appear in the main group, but had about 60% similarity with the strain URM951 of *A. sojae*. The strains URM433 and URM1873 of *A. sojae* were joined with a similarity level of 62%. The strains URM648 and URM3091 of *A. flavus* connected near *A. tamarii* at 40% similarity, and the URM5119 was joined at 25% similarity, near the strain URM3091 and the outgroup strain. The outgroup strain URM5162 of *A. niger* was correctly placed in the dendrogram.

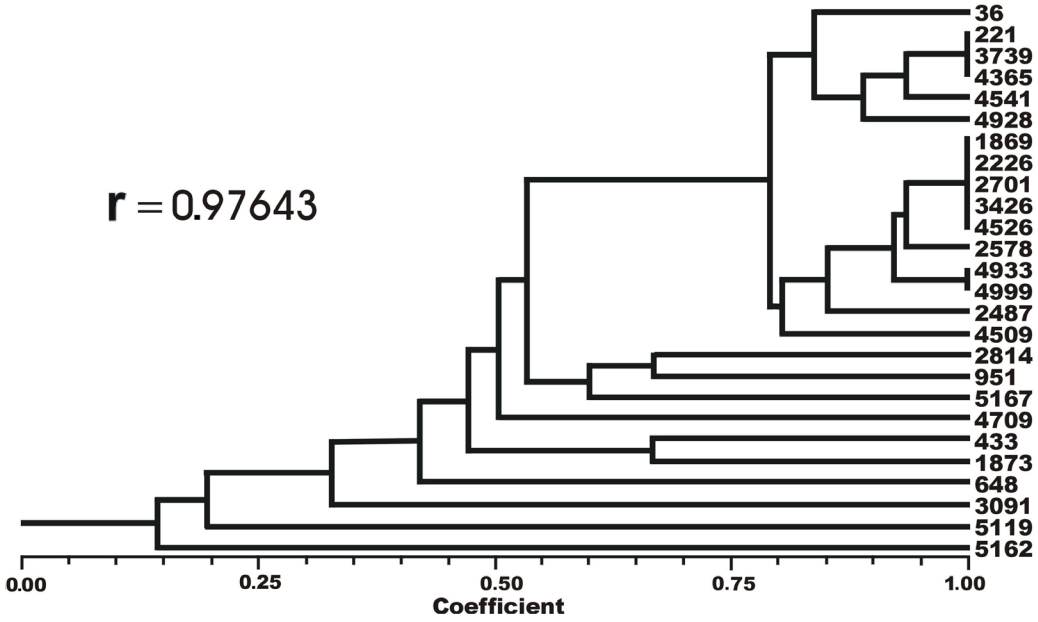

Figure 4. Dendrogram obtained by UPGMA method using Jaccard (J) similarity coefficient calculated from PCR amplification banding of ISSR with $(GTG)_5$ and $(GACA)_4$ primers of 26 strains of *Aspergillus flavus* species group. Strain accession numbers in the URM Micoteca are indicated and species classification are shown in Tables 1 and 2.

RAPD marker profiles

The RAPD banding patterns using the primers OPA-10, OPA-14, OPA-17, OPA-20, OPW-04, and OPW-05 were reproducible and displayed high genetic variability among the *A. flavus* group species. The primer OPW-04 revealed low intraspecific variability and high interspecific variability (Figure 5). The presence or absence of each band obtained with the six chosen RAPD primers was used to create a data matrix used to generate the dendrogram shown in Figure 6. The dendrogram shows the formation of two main groups with 75% similarity, containing only the *A. flavus* strains,

including the URM4933 that was suggested above to be *A. flavus* on the basis of ISSR markers. However, the *A. flavus* strains URM2814, URM648, URM4709, and URM3091 exhibited low similarity in the RAPD fragment sizes and were not grouped with the other *A. flavus* strains studied.

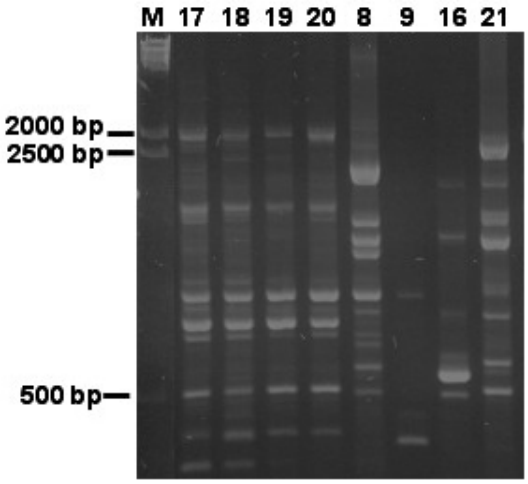

Figure 5. RAPD band profile obtained with OPW-04 of strains of *Aspergillus flavus* species group. Note the characteristic banding patterns for *Aspergillus flavus* strains 17, 19 and 20, which include also one *Aspergillus parasiticus* strain (18), and a second group of *Aspergillus flavus* strains (8, 9, 16, and 21) with a differential profile. Reclassification of species are as shown in Table 2. M = 500-bp DNA marker.

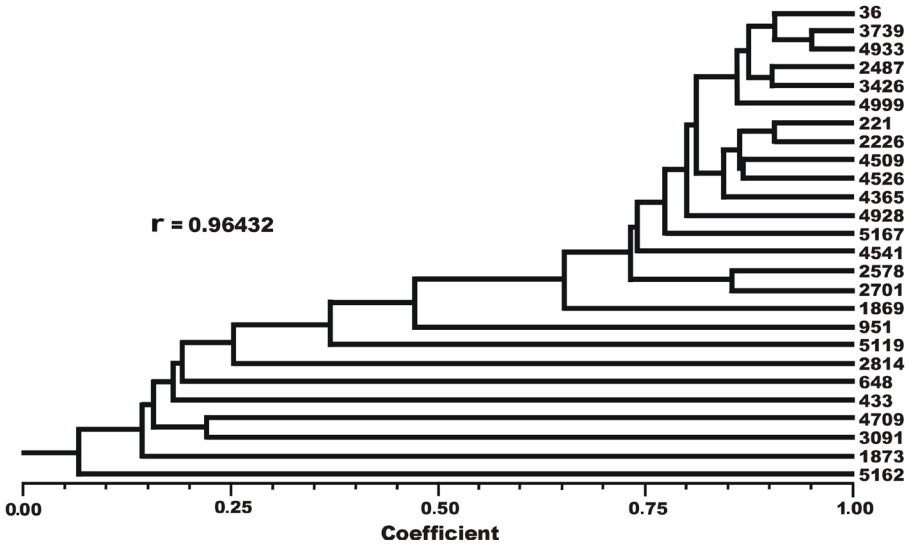

Figure 6. Dendrogram obtained by UPGMA method using Jaccard (J) coefficient calculated from RAPD band profiles obtained with OPA-10, OPA-14, OPA-17, OPA-20, OPW-04, and OPW-05 primers of 26 strains of *Aspergillus flavus* species group. Strain accession numbers in the URM Micoteca are indicated and species classification are shown in Tables 1 and 2.

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DISCUSSION

Analysis of ITS of rDNA region

Amplification of ITS1-5.8S-ITS2 with ITS4 and ITS5 primers produced a unique band of approximately 600 bp for all *Aspergillus* strains tested here (Figure 1), including the *A. niger* used as outgroup. The fragment size was as expected, since Henry et al. (2000) detected amplification fragments ranging from 565 to 613 bp for different *Aspergillus* species, which was 595 bp in *A. flavus*. The small variation in band size makes ITS an unreliable parameter for separating *Aspergillus* species (Hinrikson et al., 2005) and also inadequate for differentiating *A. flavus* strains, as also demonstrated here. The uniformity of ITS fragment size in several fungal groups makes nucleotide sequencing of ITS fragments necessary to reveal interspecific, and in some cases, also intraspecific variation (Radford et al., 1998; Turenne et al., 1999; Chen et al., 2000; Henry et al., 2000; Hinrikson et al., 2005; Inglis and Tigano, 2006). The analysis of PCR products by single-strand conformation polymorphism and restriction fragment length polymorphism allowed the classification of species in the *A. flavus* group by Kumeda and Asao (1996), given the high degree of nucleotide variation in the ITS region, undetected by PCR alone. In other fungal groups the ITS region was also very useful in resolving taxonomic difficulties, as demonstrated by Driver et al. (2000) in the taxonomic revision of *Metarhizium* and by Inglis and Tigano (2006) to reclassify entomopathogenic species of *Paecylomyces*, previously misidentified by classical methods.

PCR fingerprinting with ISSR microsatellites

The $(GTG)_5$ and $(GACA)_4$ primers produced differential amplification products, varying both in size and band intensity. Although $(GACA)_4$ revealed higher genetic variability, the number and size of $(GTG)_5$ bands were in a characteristic pattern in several strains of *A. flavus*, even though high interspecific variation was observed. Furthermore, four strains initially classified as *A. flavus* displayed differential banding patterns, which prompted us to review their taxonomic identification. After a new analysis using classical methods, two strains were reclassified as *A. tamari*, one as *A. oryzae* and one as *A. parasiticus*. On the other hand, after this analysis another strain initially classified as *A. parasiticus* that showed an ISSR banding pattern similar to that of *A. flavus* was reclassified as this species (Table 2). Characteristic banding profiles for $(GTG)_5$ were also found in strains of *Fusarium solanum* (Brasileiro et al., 2004), allowing the discrimination of all isolates. Similarly, Baleiras Couto et al. (1996) and Meyer et al. (1997) also found characteristic ISSR microsatellite profiles in strains of *Zygosaccharomyces* and *Candida*, respectively.

Table 2. Reclassification of *Aspergillus flavus* group strains after molecular analyses by RAPD and ISSR and confirmation by classical methods.

Strain	Previous classification	New classification
URM648	*Aspergillus flavus*	*Aspergillus tamarii*
URM3091	*Aspergillus flavus*	*Aspergillus tamarii*
URM4709	*Aspergillus flavus*	*Aspergillus oryzae*
URM2814	*Aspergillus flavus*	*Aspergillus parasiticus*
URM4933	*Aspergillus parasiticus*	*Aspergillus flavus*

The dendrogram produced using the ISSR data showed a main group containing exclusively *A. flavus* strains, although divided into two subgroups with a similarity level around 80% (Figure 4). The first subgroup was formed only by *A. flavus* strains isolated from different substrates in Pernambuco State, which means a high genetic similarity according to the geographical origin. The other *A. flavus* strains from several origins, including the *A. parasiticus* strain reclassified as *A. flavus*, were placed in the second subgroup. The remaining strains were included in the dendrogram with several levels of similarity, and the *A. niger* strain was properly positioned as the outgroup, since it is not a species of the *A. flavus* group. This is the first study to present the use of ISSR microsatellite markers to characterize *A. flavus* strains. Considering that it does not necessitate previous knowledge of the genome, besides being rapid and reproducible, ISSR analysis can be useful in population genetics, epidemiological surveys and ecological studies of *A. flavus*. Additionally, the $(GTG)_5$ primer can be used to generate unique products from different *Aspergillus* species that can be converted to a sequence characterized amplified region to help in taxonomic identification.

RAPD analysis

The six random primers used resulted in an RAPD profile with very different products for each *A. flavus* strain, providing evidence of its high genetic diversity. The primer OPW-04 produced a similar banding pattern in the *A. flavus* strains, except for the strains URM2814, URM4709, URM3091, and URM648. The strain URM4933, which was previously identified as *A. parasiticus* and changed to *A. flavus* on the basis of ISSR results and new classical evaluation, also had an RAPD banding pattern similar to that of *A. flavus* strains. This was similar to the report of Yuan et al. (1995) who used RAPD to differentiate *A. parasiticus* and *A. sojae*, which are morphologically similar species, and who found a strain with a divergent banding profile that was reclassified as *A. flavus* after reviewing morphological data.

The dendrogram based on RAPD profiles, shown in Figure 6, demonstrated the formation of a main group divided into two subgroups at the 75% similarity level, composed only of *A. flavus* strains, including the strain URM4933. This internal subdivision of the main group of *A. flavus* strains was also observed with ISSR data, as noted above, which makes it likely that this situation represents the high genetic variation among the strains.

However, the *A. flavus* strains URM2814, URM4709, URM3091, and URM648 showed a low genetic similarity with the main group strains, since they had a different RAPD profile. This is, further, a demonstration of the high intraspecific genetic diversity of *A. flavus*. The results shown in the present study support the use of RAPD fingerprinting for analyzing *A. flavus* strains using different primers, as demonstrated before for *A. niger* (Megnegneau et al., 1993), *A. fumigatus* (Verweij et al., 1996; Bertout et al., 2001; Bart-Delabesse et al., 2001; Lasker, 2002), *A. flavus* (Geiser et al., 1998; Diaz-Guerra et al., 2000), *A. terreus* (Rath et al., 1999), *A. nidulans* (Rath, 2001), and *A. ustus* (Rath et al., 2002). It is also true for other species, such as the fumonisin producer *Fusarium moniliforme* (Jimenez et al., 2000). The use of RAPD has allowed *A. flavus* strains to be distinguished from other species of the *A. flavus* group, reinforcing the importance for taxonomic studies, mainly to differentiate strains that show morphological variation in relation to environmental conditions.

In conclusion, the size homogeneity of amplified ITS fragment for all species of the *A. flavus* group was not effective for species identification. However, the ISSR and RAPD mo-

lecular markers made possible the detection of inter- and intraspecific genetic variation, which is actually very useful as an auxiliary tool for genetic characterization of *A. flavus* strains deposited in the URM culture collection. Correct classification is of central interest to large culture collections that provide strains for various research and biotechnological uses.

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