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Molecular Characterization of *Mucor circinelloides* and *Rhizopus stolonifer* Strains Isolated from Some Saudi Fruits

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Abstract

Thirteen strains of *Mucor circinelloides* and nine strains of *Rhizopus stolonifer* were collected from different Saudi fruits. Also, two strains of *M. circinelloides* and one strain of *R. stolonifer* isolated from soil were used in this study to analyze the genetic diversity among these 25 strains. The dendrogram constructed from random amplified polymorphic DNA–polymerase chain reaction results using two primers (V6 and M13) showed no correlation between clustering system and sources of isolation for *M. circinelloides* strains, while *Rhizopus* strains clustered according to the sources of isolation. The phylogenetic tree based on the unweighted pair group method with arithmetic average of internal transcribed spacer (ITS) rDNA sequence revealed the variation of the ITS region among *Mucor* strains as well as *Rhizopus* strains.

Introduction

Fruits and vegetables are highly perishable products, especially during the postharvest phase, when considerable losses (due to microbiological diseases, disorders, transpiration, and senescence) can occur. Losses that are related to a ready-for-marketing commodity, with a high added value, are of economic importance. The losses due to various postharvest diseases are estimated to range from 10% to 30% per year despite the use of modern storage facilities and techniques (Waller *et al.*, 2001). Fungal rots, especially in fresh fruits and vegetables, are responsible for a significant proportion of the postharvest damage. Fungi most commonly isolated as postharvest pathogens include species of *Aspergillus*, *Penicillium*, *Mucor*, and *Rhizopus*. Among the members of the order Mucorales, *Mucor piriformis*, *Gilbertella persicaria*, and various *Rhizopus* species (especially *Rhizopus stolonifer* and *Rhizopus oryzae*) are the species most frequently isolated as the causative agents of these losses. Rots caused by these fungi may be reduced by strict protective measures preventing fruit contamination during harvesting and storage (Michailides and Spotts, 1986; Michailides, 1991), but a better understanding of the biology of these microscopic fungi would be of help in the establishment of effective management strategies against these losses.

Mucor and *Rhizopus* species cause the postharvest decay of a variety of fruits and vegetables. In various instances, the *Mucor* species have been reported to have caused serious losses in strawberries, pears, apples, peaches, nectarines, guavas, tomatoes, sweet potatoes (Michailides, 1991), mangoes, and various vegetables (Smith *et al.*, 1979). Rhizopus rot caused by *R. stolonifer* is one of the most destructive diseases of stone fruits (Nishijima *et al.*, 1990; Sholberg *et al.*, 1995; Waller *et al.*, 2001; Howell, 2002; Zhang *et al.*, 2004). Rhizopus soft rot caused by *R. stolonifer* and *R. oryzae* is the most serious and widespread postharvest disease of sweet potatoes in the United States and worldwide (Holmes and Stange, 2002).

Molecular genetic approaches are now generally used for filamentous fungi too, and are fundamental for modern taxonomy (Kurtzman *et al.*, 1986; Tamura *et al.*, 1999; Voigt *et al.*, 1999; O'Donnell *et al.*, 2001; Binder and Hibbett, 2002; Moncalvo *et al.*, 2002; Lutzoni *et al.*, 2004). Molecular techniques using genomic targets within the rRNA complex have been shown to be reliable for the species identification of the zygomycetes, including *Mucor* species (Iwen *et al.*, 2002, 2005; Rakeman *et al.*, 2005; Schwarz *et al.*, 2006). RAPD analysis offers a rapid and sensitive method with which to acquire detailed information about the type and level of genetic variability (Williams *et al.*, 1991). This approach has been used efficiently for the genotypic analysis of several groups within the Zygomycetes (Burmester and Wöstemeyer, 1994; Bidochka *et al.*, 1995; Abbas *et al.*, 1996; Nelson *et al.*, 1998; Vastag *et al.*, 2000; Vágvölgyi *et al.*, 2001). Taxonomic revision of the genus *Rhizopus* has led to several independently described species made being obsolete (such as *R. reflexus*, *R. circinans*, and *R. niveus*) on morphological and physiological considerations. They are now differentiated as only two varieties within the same species: *R. stolonifer* var. *stolonifer*

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and *R. stolonifer* var. *reflexus* (Schipper, 1984). In contrast with this situation, the RAPD analysis of 29 *R. stolonifer* strains isolated from various locations and substrates revealed a high genetic heterogeneity within the species *R. stolonifer*. This suggesting practically species-level differences between the isolates of *R. stolonifer* var. *stolonifer* and *R. stolonifer* var. *reflexus* (Vágvölgyi *et al.*, 2004).

Epidemiological investigations and control of these infections require reproducible methods of distinguishing among isolates and revealing the genetic make-up of a fungal species. On the other hand, little is known about the genetic variability of these organisms, and the compositions of their populations are poorly understood. Therefore, the purpose of the present study is to characterize *Mucor circinelloides* and *R. stolonifer* species isolated from some Saudi fruits based on RAPD and ITS region of rDNA sequences to evaluate the validity of the taxonomic classification by morphological characteristics.

Materials and Methods

Fungal strains and cultivation

Twenty-two strains of Zygomycetes (13 *M. circinelloides* and 9 *R. stolonifer*) isolated from different Saudi fruits (apricot, date, grapes, and plums) were used in this study. Two strains of *M. circinelloides* and one strain of *R. stolonifer* isolated from soil were used to confirm the relation between source of isolation and DNA fingerprinting patterns. Mycelium for DNA isolation was grown in YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% dextrose; Difco, Detroit, MI) at room temperature for 2 to 5 days. Strains grown on YM agar (2% Difco agar) were examined morphologically according to the method of O'Donnell (1979) to confirm their identity.

DNA extraction

After 5 days mycelium was collected by vacuum filtration and ground to a fine powder in liquid N_2. Fifty milligrams of the powder was transferred to 1.5 mL Eppendorf tube and mixed with 700 μL CTAB buffer (0.1 M Tris–HCL, 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, and 0.2% mercaptoethanol, pH 8.0). The tubes were incubated at 65°C for 30 min, and then 700 μL of chloroform was added and the mixture vortexed briefly. The resulting mixture was centrifuged at a maximum speed of 15,000 rpm for 30 min and the cleared supernatant was mixed with 600 μL of isopropanol chilled to 72°C. The mixture was centrifuged at the maximum speed for 5 min and the resulting pellet washed twice with 1 mL of 70% ethanol. The pellet was dried under vacuum and dissolved in 100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The DNA was observed by electrophoresis in 1% agarose gel in Tris Borate EDTA buffer: 89 mM Tris–HCl (pH 8.3), 89 mM boric acid, and 20 mM EDTA (Maniatis *et al.*, 1982). Agarose gels were stained with ethidium bromide and examined under ultraviolet light.

Random amplified polymorphic DNA–polymerase chain reaction

Polymerase chain reaction (PCR) conditions and separation of random amplified polymorphic DNA (RAPD)–PCR fragments were carried out according to the techniques of Messner *et al.* (1994). PCR was carried out using primer V6 (TGCAGCGTGG; Prillinger *et al.*, 1999) and M13 (GAGGG TGGCGGTTCT; O'Donnell *et al.*, 1999), respectively. The following temperature protocol was used: initial denaturizing step of 5 min at 95°C, 40 cycles of denaturation for 15 sec at 95°C, annealing for 90 sec at 40°C, and extension for 100 sec at 72°C. The PCR products were resolved by electrophoresis by using 1.4% agarose gel in 0.5×Tris Borate EDTA buffer, at 125 V for 2 h. Gels were stained with ethidium bromide and viewed under UV light using UVP BioImaging CDS 8000 System (UVP, Cambridge, UK). Dendrograms for RAPD results were constructed by the unweighted pair group method with arithmetic average based on Jaccard's Similarity Coefficient by using Phoretix 1D Advanced v5.20 software (Non-Linear Dynamics).

ITS sequencing

Ribosomal DNA including the complete ITS1-5.8S-ITS2 region was amplified with ITS1 (TCCGTAGGTGAACCTG CGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.*, 1990). Amplification mixtures (100 μL) contained 5 μL of the extracted genomic DNA, 2.5 μL of 20 μM concentrations of each primer, 10 μL of 2.5 mM (each) dATP, dTTP, dGTP, dCTP (Roche Diagnostics GmbH, Mannheim, Germany), 10 μL of 25 mM $MgCl_2$, 6.25 U of Ampli*Taq* polymerase (Roche Diagnostics GmbH), and 10 μL of 10×PCR buffer (Roche Diagnostics GmbH). Amplification of the PCR products was done in a Bio-Rad Cycler thermocycler (Bio-Rad, Hercules, CA) with the following cycling parameters: initial denaturizing step of 10 min at 94°C, 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, and elongation for 30 sec at 72°C, with a final extension for 10 min at 72°C. PCR products were purified on P100 gel fine (Bio-Rad), and both strands were sequenced once by the Big Dye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA), with the primer set ITS1 and ITS4. Reaction products were analyzed on an ABI Prism 3700 automated DNA analyzer (Applied Biosystems).

Sequence analysis

DNA sequences were aligned with CLUSTAL Software. The Treecon program (Van de Peer and De Wachter, 1994) was used to carry out rooted parsimony analysis for investigating the tree topology. Support for the branches based on parsimony criteria estimated by bootstrap analysis of 100 replicates were generated with this program.

Results and Discussion

Twenty-five species of Zygomycetes were collected in this work (Table 1). Fifteen isolates of *M. circinelloides* and 10 isolates of *R. stolonifer* were collected from different fruits and from soil as well. Michailides (1991) isolated *M. circinelloides* from stone fruits from California and Chile. Also, Eseigbe and Bankole (1995) isolated this specie as associated fungus with postharvest rot of black plum (*Vitex doniana*) in Nigeria. *R. stolonifer* has a broad host range: many fruits and vegetables are susceptible to this pathogen—peaches, nectarines, sweet cherries, strawberries, and plums are the most susceptible. Some *Mucor* and *Rhizopus* species have been described as an important agent of postharvest fungal infections (Smith *et al.*, 1979; Michailides, 1980), causing decay in highwater-

Table 1. List of Fungal Isolates Used in This Study

Origin	*Species*	*Strain code*	*Accession no.*
Apricot	*Rhizopus stolonifer*	TUR1	AM933543
Apricot	*R. stolonifer*	TUR2	–
Apricot	*R. stolonifer*	TUR3	–
Apricot	*R. stolonifer*	TUR4	FN401528
Date	*R. stolonifer*	TUR5	AM933544
Plums	*R. stolonifer*	TUR6	AM933545
Grapes	*R. stolonifer*	TUR7	AM933546
Grapes	*R. stolonifer*	TUR8	FN401529
Soil	*R. stolonifer*	TUR9	AM933547
Soil	*R. stolonifer*	TUR10	FN421345
Apricot	*Mucor circinelloides*	TUM1	AM933548
Apricot	*M. circinelloides*	TUM2	–
Date	*M. circinelloides*	TUM3	AM933549
Date	*M. circinelloides*	TUM4	FN421346
Plums	*M. circinelloides*	TUM5	–
Plums	*M. circinelloides*	TUM6	AM933550
Plums	*M. circinelloides*	TUM7	AM933551
Plums	*M. circinelloides*	TUM8	FN401527
Plums	*M. circinelloides*	TUM9	FN421347
Plums	*M. circinelloides*	TUM10	AM933552
Grapes	*M. circinelloides*	TUM11	FN421348
Grapes	*M. circinelloides*	TUM12	AM933553
Grape	*M. circinelloides*	TUM13	FN421349
Grape	*M. circinelloides*	TUM14	AM933554
Soil	*M. circinelloides*	TUM15	AM933555

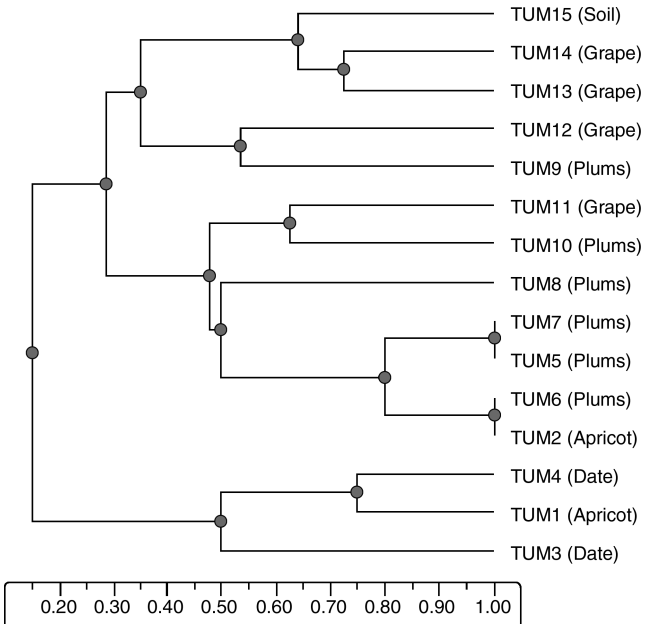

FIG. 1. Dendrogram showing relationships among 15 strains of *Mucor circinelloides* isolated from different fruits (in parentheses) and soil. Genetic distances were obtained by random amplified polymorphic DNA analysis using two different primers (V6 and M13).

content fruits (Lopatecki and Peters, 1972) and vegetables (Moline and Kuti, 1984). These infections can be significant when fruits are kept at temperature between 0°C and 20°C (Michailides, 1980).

RAPD–PCR for M. circinelloides *isolates*

Using two different primers, each isolate recorded considerable numbers of DNA patterns. The numbers of bands for M13 primer fluctuated from 11 to 17 bands. The recorded bands showed molecular weight variation from 188 to 3135 bp. On the other hand, V6 primer gave numbers of bands ranging from 13 to 19 with molecular weight ranging from 271 to 3163 for its bands. The results of M13 and V6 primers were used to construct dengrogram showing the relationships among *Mucor circinelloide* isolates (Fig. 1). RAPD analysis of 30 isolates of *M. piriformis*, collected from infected fruit, revealed 19 composite amplification types (Papp *et al.*, 1997), indicating a much higher degree of variability than found in previous isoenzyme studies (Vágvölgyi *et al.*, 1996). The dendrogram indicated that there is no correlation between clustering system and sources of isolates. For example, TUM2, TUM5, TUM6, TUM7, TUM8, TUM10, and TUM11 clustered together although they were isolated from different fruits (Fig. 1). This results may be attributed to the broad host range of *M. circinelloides*. Papp *et al.* (1997) reported that the numerical analysis of *M. piriformis* revealed three clusters, which correlated with the mating competency of the isolates or their place of origin. Their results demonstrated that RAPD analysis can identify isolates and subspecific populations of *M. piriformis*. A similar observation has been made with *Gilbertella persicaria* isolates from infected peaches and nectarines; in this case, reproducible RAPD markers were obtained which correlated with the mating types (plus or minus) of the isolates (Papp *et al.*, 2001).

RAPD–PCR for R. stolonifer *isolates*

The number of amplified bands with M13 primer varied between 15 and 18 with amplification product between 300 and 2754 bp. On the other hand the number of amplified bands varied between 12 and 18 and sizes of the detected amplification products were between 297 and 2607 bp with V6 primer. Different primers revealed different degrees of DNA polymorphism among the strains of *R. stolonifer.* Also,

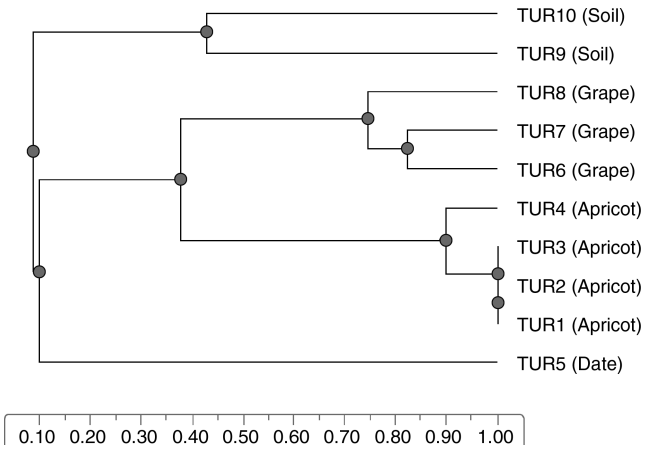

FIG. 2. Dendrogram showing relationships among 10 strains of *Rhizopus stolonifer* isolated from different fruits and soil. Genetic distances were obtained by random amplified polymorphic DNA analysis using two different primers (V6 and M13).

strains TUR1, TUR2, TUR3, and TUR4 isolated from apricot and comprised one cluster. Meanwhile TUR9 and TUR10 (isolated from soil) cluster together as shown in Figure 2. Vágvölgyi *et al.* (2004) assessed genetic variability in *R. stolonifer*, by RAPD analysis. They reported that the numerical analysis of the RAPD data revealed four main clusters with extremely high dissimilarity values, but only low or moderate variability was observed within these groups. Their results suggest a high genetic heterogeneity in the case of *R. stolonifer*: isolates of *R. stolonifer* var. *stolonifer*, *R. stolonifer* var. *reflexus*, and *R. niveus* displayed species-level genetic distances, which gives rise to considerations that they might be separate species. A dendrogram was constructed from these data by using unweighted pair group method with arithmetic average method. The *R. stolonifer* strains formed three different clusters. The clustering system followed the source of isolation. Strains TUR6, TUR7, and TUR8 isolated from grape and constituted one cluster (Fig. 2). Vágvölgyi *et al.* (2004) reported that three *R. stolonifer* isolates isolated from infected nectarines and peaches belonged to one cluster. These isolates exhibited similar morphological characteristics and growth requirements as other *R. stolonifer* isolates, although they displayed a very few common RAPD markers with isolates in the other clusters. The work by Vágvölgyi *et al.* (2004) supports the need for a reconsideration of the original taxonomic distinction.

Sequencing

Since TUM2 and TUM6; TUM5 and TUM7; and TUR1, TUR2, and TUR3 were completely identical according to their RAPD results; TUM6, TUM7, and TUR1 were used as representative strains for sequencing. The other isolated strains were applied for sequencing analysis. The phylogenetic tree was made with those data and some known sequences from the databases (Fig. 3). Among these DNA sequences, a major difference that could divide the sequences into two groups was detected (Fig. 3). According to the phylogenetic tree constructed from the sequences results there were two different clades; *Mucor* clade and *Rhizopus* clade.

Mucor clade was divided into two subclades. The first subclade comprised of seven strains (from TUM9 to TUM15). These strains clustered together with *M. circinelloides* (DQ837219) from GenBank with 79 bootstrap values. On the

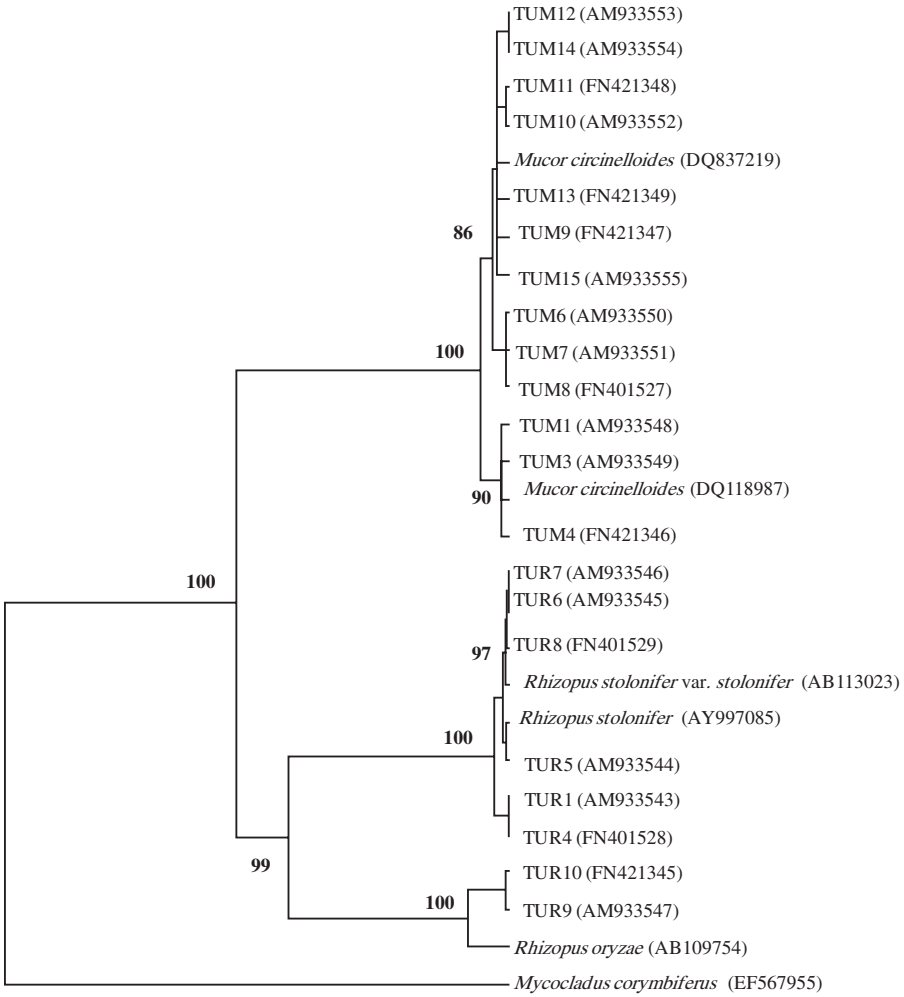

FIG. 3. The unweighted pair group method with arithmetic average phylogenetic tree or rDNA ITS sequences of *Mucor* and *Rhizopus* strains. Bootstrap percentage over % from 100 replicates are shown in each branch. The number in parentheses indicated their database accession numbers.

other hand, the second subclade included six strains (TUM1, TUM3, TUM4, TUM6, TUM7, and TUM8) and *Mucor circinellonides* (DQ118987) from GenBank with 80 bootstrap values. These results indicated the variation of ITS rDNA in *M. circinelloides* strains. Nagy *et al.* (2000) reported that for the *M. circinellonides* and *Mucor racemosus* strains, extensive chromosome-length polymorphism was observed, whereas the karyotypes of the *Mucor plumbeus* were much more similar. This phenomenon may somehow reflect that both *M. circinellonides* and *M. racemosus* species unify several species formae in the current taxonomic approach, while *M. plumbeus* is a single specie with well-defined morphological characteristics (Schipper, 1976). This explanation is supported by the results of earlier isoenzyme studies on 27 *Mucor* strains; for *M. circinellonides* and *M. racemosus*, much higher levels of intraspecific variability were found than for *M. plumbeus* (Varga and Vágvölgyi, 1991). Schipper (1976) described four formae (*M. circinelloides* f. *circinelloides*, *M. circinelloides* f. *janssenii*, *M. circinelloides* f. *griseocyanus*, and *M. circinelloides* f. *lusitanicus*) based on differences in the shapes of spores and columellae and in thermotolerance. Schwarz *et al.* (2006), and confirmed by Iwen *et al.* (2007), showed that *M. circinelloides* demonstrates variability within the ITS regions, potentially supporting subspecies groups.

Rhizopus clade was divided into two subclades: *R. oryzae* group subclade and *R. stolonifer* subclade. The first one comprised of strains TUR9 and TUR10 that had the same sequence of *Rhizopus oryzae* AB109754. These strains were identified mistakenly (based on morphological criteria) as *R. stolonifer* but it is clear now that they were *R. oryzae* according to the sequence results. Vágvölgyi *et al.* (2004) reported that isolates of *R. oryzae* from very different origin formed a well-defined group, supporting the unity of this species.

R. stolonifer subclade included strains TUR1 and TUR4 that clustered together with *R. stolonifer* AY625072 with 69 boostrap values. While TUR5 and TUR8 clustered together with *R. stolonifer* AY997085 with 89 boostrap values. This clustering system supports the heterogeneity of the ITS region among *R. stolonifer* strains. Schipper and Staplers (1984) divided the genus *Rhizopus* into three groups; the *stolonifer*, *oryzae*, and *microsporus* groups based on the general morphology and mating experiments. However, the morphological and physiological characteristics of these species tend to overlap species lines. Our results came in agreement with Abe *et al.* (2006) findings. They sequenced three molecules of the ribosomal RNA encoding DNA (rDNA), complete 18S, ITS1-5.8S-ITS2, and 28S D1/D2 regions of all the species of the genus *Rhizopus*. Their phylogenetic trees showed three major clusters corresponding to the three groups in the current morphological taxonomy: microsporus group, stolonifer group, and *R. oryzae*.

In conclusion our results indicate the variation of ITS region sequence among *M. circinelloides* strains and also possibility of the molecular identification of the *Rhizopus* species groups using rDNA sequencing.

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Disclosure Statement

No competing financial interests exist.

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