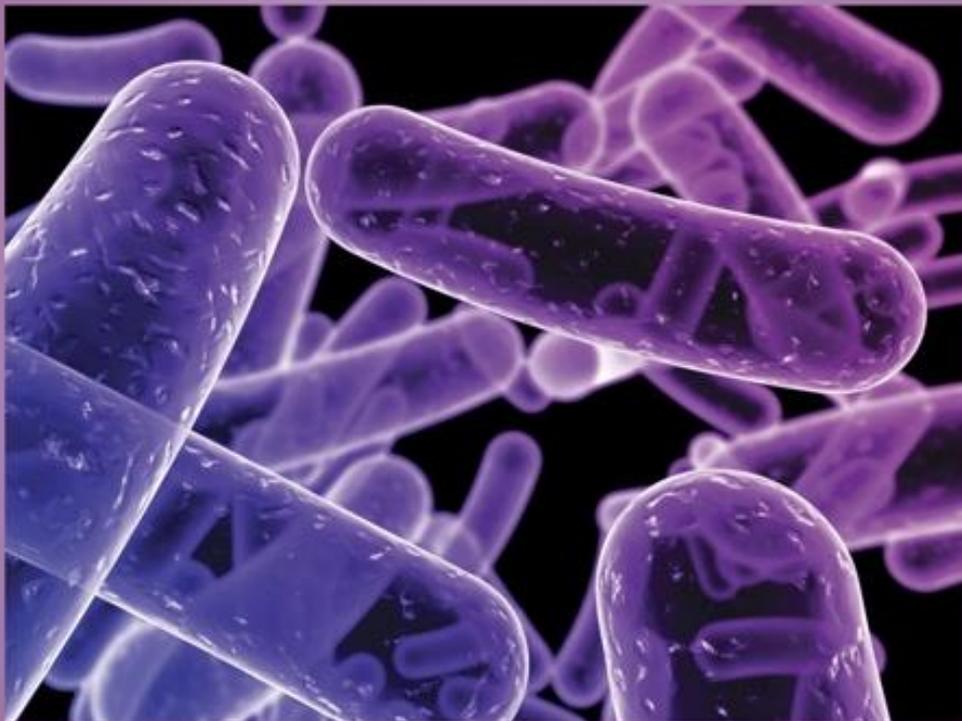




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Genetic Diversity of Mold-Causing Fungi in Strawberry and Orange Fruits Using RAPD Markers

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ABSTRACT

The purpose of this study is to investigate the intra- and inter-specific genetic relationship of some strawberry- and orange-associated fungi based on DNA markers technique. Five fungal isolates belonging to three different genera were isolated from strawberry and orange samples. The isolates were morphologically characterized as *Aspergillus fumigatus*, which originated from orange samples; *A. flavus* and *A. niger*, both originated from strawberries; and *Rhizopus stolonifer* and *Alternaria alternata*, both originated from orange samples. The five fungal isolates were subjected to investigating intra- and inter-specific genetic relatedness, employing the technique of random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) marker using ten oligonucleotide primers. The RAPD-PCR analysis revealed a high interspecific relationship between the three *Aspergillus* species and *Rhizopus stolonifer*, as well as *Alternaria alternata*. It also revealed a high intraspecific genetic relationship between *A. fumigatus* and *A. flavus*, which were isolated from different environments, surpassing the observed relationships between *A. flavus* and *A. niger*, which were isolated from the same environment. This finding confirms genetic homogeneity within the studied *Aspergillus* species, suggesting a potential for molecular identification of genetic diversity among different individuals in the *Aspergillus* genus, taking into consideration their respective origins.

INTRODUCTION

Fungi play crucial roles in ecological processes, including nutrient cycling, maintaining plant and animal health, organic decomposition, environmental protection, and forming mutualistic symbioses with plants, algae, and cyanobacteria (Gadd 2013; Grossart *et al.* 2016). However, they can also act as significant post-harvest pathogens (Pan *et al.* 2014; Li *et al.* 2017).

Applied mycology seeks to minimize fungal damage, especially in the context of food (Nout 1995; Lange 2014). The growth of fungi in food may result in the production of harmful mycotoxins, negatively impacting human health (May and Adams 1997; Pitt 2000). Accurate identification of fungi at the species level is essential for various research domains, including taxonomy, biodiversity, ecology, plant pathology, and health (Martin *et al.* 2011; Araujo 2014; Xu 2016). As well as advanced methods using DNA markers, such as random amplified polymorphic DNA (RAPD), inter simple sequences repeat (ISSR), and start codon targeted (SCoT) DNA markers, are employed to assess intra- and inter-specific genetic diversity within fungal communities.

Since 2012, RAPD has been widely applied in exploring fungal genetic diversity, investigating various plant pathogens, and ensuring the authenticity and safety of products such as food and beverages. This technique utilizes short random PCR primers to target diverse genomic regions, generating distinct PCR profiles for each species (Bardakci 2001) and employing highly polymorphic molecular markers, requires a moderate DNA quantity and entails intermediate technical and operational expenses (Welsh and McClelland 1990; Williams *et al.* 1990). Here, we aim to investigate the genetic relationships between five isolated fungal species that cause strawberry and orange fruit mold using the RAPD-PCR technique.

MATERIALS AND METHODS

Source of the Five Fungal Isolates:

The five fungal isolates under investigation were isolated and morphologically identified at the Plant Clinic Unit, Faculty of Agriculture, Ain Shams University. Briefly, the isolates were isolated from strawberry and orange fruit samples collected from Souq El Obour and exhibited mold. The morphological identification was conducted through the

modified micro chamber agar spot slide culture method developed by Prakash and Bhargava (2016), employing light microscopy and a potato dextrose agar (PDA) medium and also identified by comparing their cultural and morphological features with published descriptions (Brooks 1931; Howard *et al.* 1983; Smith and Black 1990).

Genetic Diversity of the Five Fungal Species:

Genomic DNA Extraction:

Five selected fungal isolates were subjected to DNA extraction according to the method described by Al-Samarrai and Schmid (2000).

RAPD-PCR Reaction:

The amplification reaction was performed using ten random oligonucleotides listed in Table 1, rich with GC content (60-70%) and belonging to different Operons. RAPD-PCR reaction was performed following the method described by Williams *et al.* (1993). Each reaction was executed in a final volume of 25 μ L containing 2.5 μ L of 10X PCR buffer (500 Mm KCl, 100 mM TrisHCl (pH = 9.0), 1% Triton-100), 1.5 μ L of 1.5 mM MgCl₂, 0.5 μ L of dNTPs mix (0.2 mM dATP, dCTP, dTTP, dGTP), 1.0 μ L of DNA template (100 ng genomic DNA), 1.5 μ L of 0.4 μ M decamer oligonucleotide primer, 0.5 μ L of *Taq* DNA polymerase (two units of *Taq* DNA polymerase-Promega Crop, Madison, WI, USA), and 17.5 μ L of sterile d.H₂O.

PCR amplification was carried out in a thermocycler (Eppendorf) employing a GeneAmp 2400 PCR machine, utilizing the following program: initial denaturation at 94°C for 5 minutes, followed by 35 cycles comprising denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes. Additionally, a final extension step was included, involving one cycle at 72°C for 7 minutes and subsequently cooled to 4 °C. The RAPD products were separated through electrophoresis on a 1.5% agarose

gels in 1X Tris-borate-EDTA (TBE) buffer at a voltage of 70 V for one and a half hours, employing the DNA 100 bp Plus DNA Ladder as a size reference. Ethidium bromide at a concentration of 0.01% was

used for staining, and the products were visualized by the UV light transilluminator, and the resulting images were digitally recorded for subsequent analysis (Sambrook *et al.* 1989).

Table 1: Random oligonucleotides used in RAPD-PCR analysis of the five fungal isolates and their operons, sequences, G+C and A+T contents.

Operons	Primers No.	Sequences (5'..... 3')	GC (%)	
			A+T	G+C
OPA	01	CAGGCCCTTC	30	70
	02	TGCCGAGCTG	30	70
OPB	04	GGAGTGGAGT	40	60
	10	GGTGACGCAG	30	70
OPC	08	TGGACCGGTG	30	70
	11	AAAGCTGCGG	40	60
OPD	11	AGCGCCATTG	40	60
OPE	18	GGACTGCAGA	40	60
OPG	18	GAGCCCTCCA	30	70
OPH	04	GGAAGTCGCC	30	70

RAPD Analysis:

All the amplified DNA fragments generated by RAPD-PCR using the ten primers were considered and analyzed separately to determine the fragment type. The amplified fragments were recorded as 1 (If present) and 0 (If absent), and the fragments were classified into monomorphic and polymorphic. The similarity coefficient (F) between isolates was defined by formula of Nei and Li (1979). Cluster analysis was performed using the unweighted pair group method with the arithmetic mean (UPGMA) method contained in the computer program package NTSYS 1.5 (Rohlf 1990). The relationships between the pattern profiles are displayed as dendrogram and expressed as percentage similarity.

RESULTS AND DISCUSSION

Fungi play a prominent role in recycling energy and nutrients, making

them vital microorganisms. However, applied mycology focuses on mitigating damage caused by fungi, particularly in the context of food (Nout 1995; Lange 2014). Fungus growth in food can lead to the production of harmful mycotoxins, adversely affecting human health (May and Adams 1997; Pitt 2000). Strawberries and oranges are important examples of the most famous fruits exhibited to infect mold fungi in Egypt. Here, we focused our attention on investigating the genetic relationships between some post-harvest fungus pathogens isolated from strawberries and oranges. Three isolates identified as *Aspergillus fumigatus*, *Rhizopus stolonifer*, and *Alternaria alternata*, obtained from the orange samples, and two isolates as *Aspergillus flavus* and *Aspergillus niger*, obtained from the strawberry samples (Fig. 1).

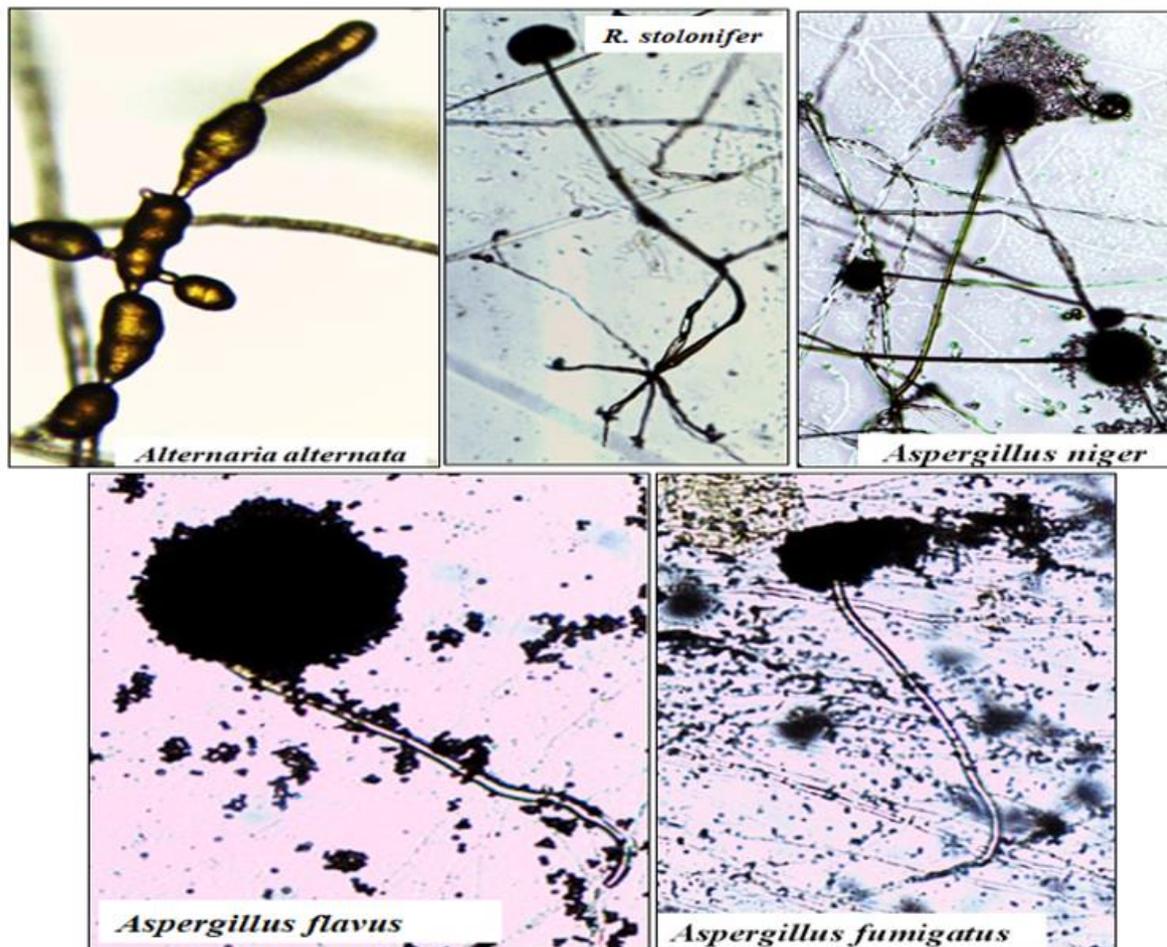


Fig. 1: Morphological characteristics of the five fungal species using the modified micro chamber agar spot slide culture method.

RAPD Analysis for Genetic Relationships Between the Five Fungal Isolates:

The RAPD-PCR technique is considered a highly valuable molecular tool for assessing the genetic diversity of microbial strains because of its cost-effectiveness, methodological simplicity, rapidity, safety, high sensitivity, and the comprehensive nature of the results obtained (Ikeh 2003).

A total of 133 scorable fragments were produced by ten random oligonucleotide primers from five fungal species, namely, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Alternaria alternata*. Out of them, seventy-nine fragments were found to be polymorphic, ranging from 3 to 11 fragments with an average of 7.9 fragments per primer, and a polymorphism

average of 58.9%. In contrast, fifty-four fragments were found to be monomorphic, ranging from 3 to 9 fragments with an average of 5.4 fragments per primer, and a monomorphism average of 41.05% (Table 2). The number of total amplified DNA fragments (TADFs) produced by each primer ranged from 11 fragments with primers OPA01, OPG18, OPH04 to 17 fragments with primer OPC11, with an average of 13.3 fragments per primer. Among these primers, OPB10 yielded the highest number (11) of polymorphic fragments with polymorphism of 78.57%. While the primers OPH04 yielded the highest number (8) of monomorphic fragments representing 72.73% monomorphism (Table 2). Amplification products produced by these primers ranged from 100 bp to 3000 bp in size, using a standard 100 bp Plus DNA ladder as a

marker (Fig. 2). The diverse numbers of fragments generated by each primer could be attributed to variations in sequences, the presence of annealing sites in the genome, and the quality of the template, as noted by Kernodle *et al.* (1993).

Across the five fungal species studied, the produced TADFs ranged from 106 to 90 fragments, which were yielded by *Aspergillus niger* and by *Alternaria*

alternata, respectively. Generally, *Aspergillus* species produced the highest number of TADFs compared to the other tested fungal species (Table 2). The highest TADFs among them were revealed by *Aspergillus niger* (106 fragments, 79.69%), followed by *Aspergillus fumigatus* and *Aspergillus flavus* (100 fragments for each, 75.19%).

Table 2: RAPD-PCR characteristics of the five fungal species generated by ten random oligonucleotide primers.

Primers	TADFs	Monomorphic bands	Polymorphic bands	Monomorphism %	Polymorphism %	Total amplified fragments (TADFs) of fungal isolates				
						<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. niger</i>	<i>R. stolonifer</i>	<i>A. alternata</i>
OPA01	11	3	8	27.27	72.73	9 (81.8%)	4 (36.36%)	8 (72.73%)	9 (81.82%)	8 (72.73%)
OPA02	16	5	11	31.25	68.75	11 (68.75%)	14 (87.50%)	10 (62.50%)	10 (62.5%)	10 (62.5%)
OPB04	13	5	8	38.46	61.54	9 (69.23%)	8 (61.54%)	10 (76.92%)	9 (69.23%)	11 (84.61%)
OPB10	14	3	11	21.42	78.57	8 (57.14%)	8 (57.14%)	8 (57.14%)	7 (50.00%)	4 (28.57%)
OPC08	13	9	4	69.23	30.77	13 (100%)	13 (100%)	12 (92.31%)	11 (84.62%)	10 (76.92%)
OPC11	17	7	10	41.18	58.82	11 (64.71%)	11 (64.71%)	15 (88.24%)	12 (70.59%)	12 (70.59%)
OPD11	12	5	7	41.67	58.33	9 (75.00%)	9 (75.00%)	10 (83.33%)	11 (91.67%)	8 (66.67%)
OPE18	15	6	9	40.00	60.00	10 (66.67%)	13 (86.67%)	12 (80.00%)	12 (80.00%)	10 (66.67%)
OPG18	11	3	8	27.27	72.73	10 (90.91%)	10 (90.91%)	10 (90.91%)	4 (36.36%)	9 (81.82%)
OPH04	11	8	3	72.73	27.27	10 (90.91%)	10 (90.91%)	11 (100%)	11 (100%)	8 (72.73%)
Mean	13.3	5.4	7.9	41.05	58.9	10	10	10.6	9.6	9.00
Total	133	54	79			100 (75.19%)	100 (75.19%)	106 (79.69%)	96 (72.18%)	90 (67.67%)

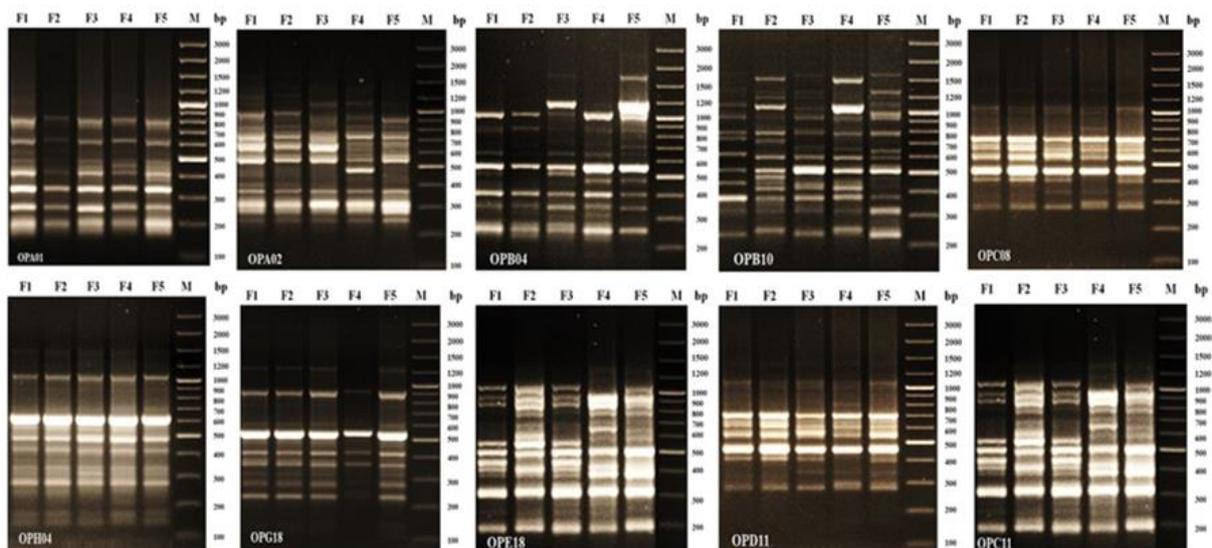


Fig. 2: RAPD-PCR profile of the five fungal species generated with ten random oligonucleotide primers. M, 100 bp Plus DNA Ladder. *A. fumigatus* (F1), *A. flavus* (F2), *A. niger* (F3), *R. stolonifer* (F4) and *A. alternata* (F5).

Concerning the similarity indices among the five fungal species (Table 3), the highest intraspecific genetic similarity index, at 79.8%, was noted between *Aspergillus fumigatus* and *A. flavus*. Despite being isolated from distinct environments (orange and strawberry samples, respectively), this result suggests a close relationship between these two species. Similarly, a significant similarity index of 77.8% was observed between *A. fumigatus* and *A. niger*, both also isolated from different environments (orange and strawberry samples, respectively). However, a lower intraspecific genetic similarity index of 66.9% was found between *A. flavus* and *A. niger*, originating from the same environment (strawberry samples). This observation aligns with the clustering pattern of *Aspergillus* species (Fig. 3), highlighting a species-dependent rather than environment-dependent (source of isolation) clustering pattern in this study.

In contrast to our findings, Gherbawy and Hussein (2010) used RAPD markers to differentiate 22 strains of *Mucor circinelloides* and *Rhizopus stolonifer* from different fruits in KSA. Their results showed the clustering of *Rhizopus* strains based on isolation sources, suggesting a correlation between the source of isolation and the clustering pattern of *Rhizopus* strains. The observed discrepancy could stem from inherent differences between the genera *Rhizopus* and *Aspergillus*, which

were utilized in both studies. Underscoring the capability of the RAPD-PCR technique to discern intra-specific genetic similarities, irrespective of the species' origin (source of isolation).

Regarding the inter-specific genetic diversity, the similarity index varied from 60.6% between *Alternaria alternata* and *Rhizopus stolonifer* to 70.4% between *Alternaria alternata* and *A. fumigatus*. The other two *Aspergillus* species, *A. flavus* and *A. niger* exhibited similarity values ranging from 63.8% to 66.4%, respectively, when compared to *Rhizopus stolonifer* and from 66.1% to 67.4%, when compared to *Alternaria alternata* (Table 3). Our findings align with studies conducted by Al-Wadai *et al.* (2013), who utilized RAPD- and ISSR-PCR techniques to assess genetic diversity among 12 fungal species across *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* genera. Their findings highlighted significant genetic diversity, particularly among *A. flavus* isolates. Similarly, Rassin *et al.* (2015), investigated genetic variations in seven isolates of *Aspergillus fumigatus* using RAPD-PCR and ISSR methods. Additionally, Valencia-Ledezma *et al.* (2022) differentiated between *A. tubingensis*, *A. fumigatus*, *A. flavus*, and *A. niger* using RAPD-PCR with 34 primers, successfully distinguishing between the species using four specific primers.

Table 3: Similarity matrix of DNA polymorphisms of the five fungal species under investigation generated among RAPD-PCR technique using ten different random oligonucleotides as primers.

Fungal species	<i>Aspergillus fumigatus</i>	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Rhizopus stolonifer</i>	<i>Alternaria alternata</i>
<i>Aspergillus fumigatus</i>	100				
<i>Aspergillus flavus</i>	79.8	100			
<i>Aspergillus niger</i>	77.8	66.9	100		
<i>Rhizopus stolonifer</i>	59.3	63.8	66.4	100	
<i>Alternaria alternata</i>	70.4	66.1	67.4	60.6	100

According to a dendrogram constructed using UPGMA based on Jaccard's similarity coefficients dependent on genetic similarity, inter and intra-species diversity ranged from 0.60 to 1.00 (Fig. 3). The dendrogram showed that the three *Aspergillus* species could be grouped into two clusters with about 75% genetic similarity. The first cluster consisted of *A. fumigatus* and *A. flavus*, while the

second was formed by *A. niger*. Suggesting that *A. fumigatus* and *A. flavus* are the most closely related species within the tested *Aspergillus* species in this study as they grouped. While *A. niger* is less related. Additionally, the lowest similarity was detected between the three *Aspergillus* species and *Rhizopus stolonifer*, as well as between *Alternaria alternata* and *Rhizopus stolonifer*.

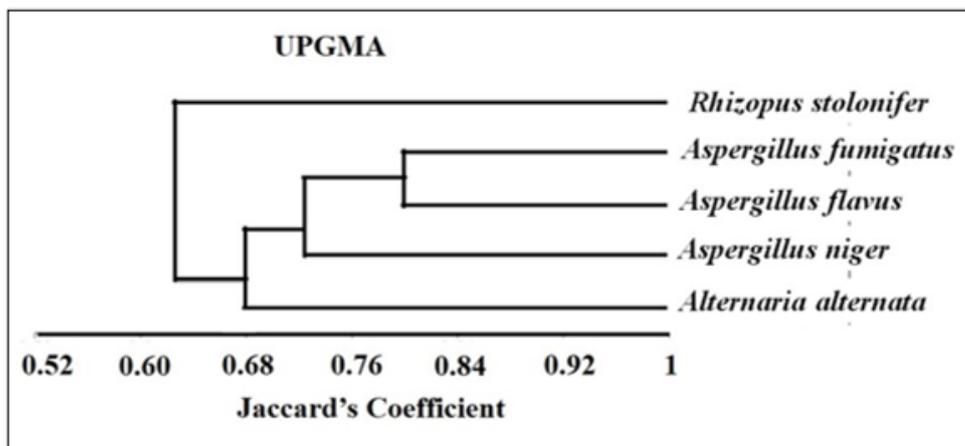


Fig. 3: Dendrogram of genetic similarity and inter- and intra-species diversity of the *Aspergillus* species and the other ones.

Conclusion

The RAPD-PCR analysis showed a high degree of intra-specific genetic relationship between *Aspergillus fumigatus* and *A. flavus*, which originated from different environments, compared to those observed between *A. flavus* and *A. niger*, which originated from the same environment. This, in turn, confirms the importance of such molecular tool, *i.e.*, RAPD-PCR, for evaluating the genetic homogeneity of the examined species of the genus *Aspergillus* and might create an opportunity for other molecular methods for identification of the genetic diversity within the different individuals within the same genus.

Declarations:

Ethical Approval: It is not applicable.

Conflicts of Interest: The authors declare that they have no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page

have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

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