http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2016.143.157

Research Article
Characterization of *Cladosporium* Species by Internal Transcribed Spacer-PCR and Microsatellites-PCR

[1]Mohammed S. Alhussaini, [2]Mohamed Adbo Moslem, [3]Mohammed I. Alghonaim, [1]Abdullah A. Al-Ghanayem, [1]Abdulrahman A.I. AL-Yahya, [1]Hamido M. Hefny and [1]Adbul Moneam Saadabi

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Saudi Arabia
[2]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[3]Department of Biology, College of Science and Humanities, Al-Quway'iyah, Shaqra University, Saudi Arabia

Abstract

Background: This investigation compared genetic similarities and diversities within and among *Cladosporium* species populations using the two PCR-based markers; Internal Transcribed Spacer (ITS)-PCR and microsatellite-PCR. **Methodology:** Nuclear ribosomal DNA internal transcribed spacers have been used to analyze intraspecific and interspecific relationships in various fungi. In the present study, the internal transcribed spacer (ITS)-PCR and microsatellite-PCR were used to identify the genetic diversities in *Cladosporium* species. **Results:** The Internal Transcribed Spacer (ITS) was amplified using polymerase chain reaction combining primers ITS4 and ITS5. The PCR products were digested with three restriction enzymes and separated by agarose gel electrophoresis. Restriction patterns generated by *Cfo*I and *Msp*I and *Rsa*I were unique for most species assayed. The ITS-PCR fingerprinting methods led to a clear differentiation of the isolates at the species level. Fingerprinting profiles generated readily discriminated between each of the 6 species. Cluster analysis further supported this observation and clusters corresponding to each species were readily identified in the dendrograms. Seven microsatellite primers out of eight primers were unable to generate visible DNA fingerprints. **Conclusion:** Amplification experiments demonstrated that microsatellite primer, T3B and $(GTG)_5$ are technically simple tools for assaying genetic variability in *Cladosporium* spp.

Key words: ITS-PCR, microsatellite, Cladosporium, DNA fingerprinting, *Cfo*I, *Msp*I, *Rsa*I

Received: December 30, 2015 Accepted: February 25, 2016 Published: March 15, 2016

Citation: Mohammed S. Alhussaini, Mohamed Adbo Moslem, Mohammed I. Alghonaim, Abdullah A. Al-Ghanayem, Abdulrahman A.I. AL-Yahya, Hamido M. Hefny and Adbul Moneam Saadabi, 2016. Characterization of *Cladosporium* species by internal transcribed spacer-PCR and microsatellites-PCR. Pak. J. Biol. Sci., 19: 143-157.

Corresponding Author: Mohammed S. Alhussaini, Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Saudi Arabia Tel: +966-569506939

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA) repeat unit are well-investigated sequences that are present in multiple copies and can be easily isolated by PCR amplification. The ITS regions usually vary between species within a genus. Hence, they have been used in numerous phylogenetic studies of various fungi[1]. Analysis of the ITS region is used to distinguish species and higher taxonomic divisions[2]. The DNA fingerprint has been used to study several genera. Restriction Fragment Length Polymorphism (RFLP) of the ITS region is used to distinguish between species within same genera and also species from different genera[3].

Microsatellites, also known as Short Tandem Repeats (STRs) and Simple Sequence Repeats (SSRs) are tandem repeats of simple dinucleotide, trinucleotide, tetranucleotide, pentanucleotide or hexanucleotide sequence (two to six bases) that occur abundantly and at random in most eukaryotic genomes[4]. They are typically short and embedded within a unique sequence, thus being ideal for designing flanking primers for *in vitro* amplification by the Polymerase Chain Reaction (PCR)[5]. Microsatellite markers can be used to detect polymorphism in plants, fungi and animal genomes[6,7]. The stability displayed by microsatellites make them perfect markers for use in constructing high-resolution genetic maps to identify susceptibility loci involved in common genetic diseases. They can be used for generating attractive molecular markers that can show codominant inheritance, multi-allelism[8] and random distribution throughout the genomes[9].

The aim of this study is to explore the genetic variation between various isolated Cladosporium strains using the Internal Transcribed Spacer-Polymerase Chain Reaction (ITS-PCR) and microsatellites (MP-PCR).

MATERIALS AND METHODS

Fungal isolates: The ITS-PCR and MP-PCR techniques were performed in 23 fungal isolates. Four reference isolates brought from Central bureau; voor Schimmelcultures, Utrecht (The Netherlands) were used as control; *Cladosporium cladosporioides* (CBS 131.29), *C. herbarum* (CBS 673.69), *C. sphaerospermum* (CBS 114326) and *C. tennuissimum* (CBS 117134). The other 19 fungal isolates, Clad#1 through Clad#19, were four isolates of *C. cladosporioides* (Fresenius) de Vries, seven isolates of *C. sphaerospermum* Penzig, five isolates of *C. herbarum* (Persoon) Link, two isolates of *C. macrocarpum* Preuss and one isolate of *C. chlamydosporis* Matsushima. The order of the isolates that resulted in the dendrograms are described in Table 1.

Fungal culture and DNA preparation: Genomic DNA was extracted from 5-7 days old cultures of the fungal isolates, grown in micro-culture tube containing 3 mL of malt extract broth at 25°C. Mycelia were harvested by filtration through two layers of cheesecloth and washed with sterile distilled water. Samples were frozen in liquid nitrogen and were ground to fine powder using mortar and pestle. The DNA was extracted using a modification of the traditional Sodium Dodecyl Sulphate (SDS) extraction procedure reported by Abd-Elsalam *et al.*[10].

Internal Transcribed Spacer-PCR (ITS-PCR) conditions: The PCR was conducted in a 25 µL reaction volume. Three microliters of DNA template (1 ng quantified using a spectrophotometer) was added to a 5 µL master mix of thermostable DNA polymerase (Jena Bioscience GmbH, Cat. No. PCR-101S), 13 µL of PCR grade water (Jena Bioscience GmbH), 2 µL of 10 pmol of ITS4 primer and 2 µL of 10 pmol of ITS5 primer (GE Healthcare). The thermal cycling parameters were carried out using a thermal cycler (Techne TC-312, Techne, Stone, UK) as follows; initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, then final extension at 72°C for 10 min. The PCR product was checked on 1.5% agarose containing 0.05 µg mL^{-1} ethidium bromide. Photographs were taken after 30 min[10].

ITS-PCR product digestion with restriction enzymes: Internal transcribed spacer fragments were digested using three base cutter restriction enzymes: *Cfo*I, *Msp*I and *Rsa*I. Fifteen microliters of amplified DNA was digested for 2 h at 37°C using a DNA thermal cycler (Techne TC-312, Techne, Stone, UK) as follows; 15 µL of ITS-PCR product, 1 µL of enzyme (1 U), 2.5 µL of buffers provided by the manufacturer and 2.5 µL of PCR grade water (Jena Bioscience GmbH). The resulting DNA fragements were electrophoresed on 1.7% agarose containing 0.05 µg mL^{-1} ethidium bromide (Agarose 25). The DNA patterns were visualized and photographed under ultraviolet light (UV)[11].

Table 1: Order of the isolate, isolate code AUMC number and CBS number of *Cladosporium* spp.

Isolate code	*Cladosporium* spp.	AUMC No.	CBS No.
CC CBS	*Cladosporium cladosporioides* (Fresenius) de Vries		CBS 131.29
Clad#1	*Cladosporium cladosporioides* (Fresenius) de Vries	4432	
Clad#8	*Cladosporium cladosporioides* (Fresenius) de Vries	4439	
Clad#14	*Cladosporium cladosporioides* (Fresenius) de Vries	4445	
Clad#19	*Cladosporium cladosporioides* (Fresenius) de Vries	4450	
CS CBS	*Cladosporium sphaerospermum* Penzig		CBS 114326
Clad#2	*Cladosporium sphaerospermum* Penzig	4433	
Clad#4	*Cladosporium sphaerospermum* Penzig	4435	
Clad#9	*Cladosporium sphaerospermum* Penzig	4440	
Clad#10	*Cladosporium sphaerospermum* Penzig	4441	
Clad#13	*Cladosporium sphaerospermum* Penzig	4444	
Clad#17	*Cladosporium sphaerospermum* Penzig	4448	
Clad#18	*Cladosporium sphaerospermum* Penzig	4449	
CH CBS	*Cladosporium herbarum* (Persoon) Link		CBS 673.69
Clad#3	*Cladosporium herbarum* (Persoon) Link	4434	
Clad#5	*Cladosporium herbarum* (Persoon) Link	4436	
Clad#6	*Cladosporium herbarum* (Persoon) Link	4437	
Clad#7	*Cladosporium herbarum* (Persoon) Link	4438	
Clad#11	*Cladosporium herbarum* (Persoon) Link	4442	
Clad#12	*Cladosporium macrocarpum* Preuss	4443	
Clad#15	*Cladosporium macrocarpum* Preuss	4446	
Clad#16	*Cladosporium chlamydosporis* Matsushima	4447	
CT CBS	*Cladosporium tenuissimum* Cooke		CBS 117134

AUMC: Assiut University, Mycological Centre

Microsatellite-PCR (MP) conditions: Microsatellite-PCR was conducted in a 25 µL reaction volume. Three microlters of DNA template (1 ng quantified using spectrophotometer) was added to a 7 µL master mix of thermostable DNA polymerase (Jena Bioscience GmbH), 13 µL of PCR grade water (Jena Bioscience GmbH), 2 µL of 20 pmol Microsatellite primer. Microsatellite primers used were; $(AGG)_5$, $(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(GTGC)_4$, $(AG)_8C$ and T3B. Primers were brought from Metabion International AG GmbH. The thermal cycling parameters were as follows; initial denaturation at 94°C for 4 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min and final extension was achieved at 72°C for 7 min. The PCR product was checked on an 1.5% agarose containing 0.05 µg mL^{-1} ethidium bromide photographs were taken after 30 min[10].

RESULTS

The current investigation evaluated the potential of DNA fingerprinting molecular marker techniques to discriminate Cladosporium isolates.

Molecular characterization and genetic diversity of *Cladosporium* species as revealed by Internal Transcribed Spacer (ITS) PCR

Internal Transcribed Spacer (ITS) PCR patterns of 23 *Cladosporium* spp. isolates digested with *Cfo*I: The ITS-PCR fingerprint patterns were analyzed using PC-windows software; BandMap package. Following conversion, normalization and background subtraction by using mathematical algorithms, levels of genetic similarity between ITS-PCR fingerprints were calculated by Pearson product-moment correlation coefficient. A cluster analysis was performed by using the UPGMA algorithm. The genetic similarity between Cladosporium isolates ranged from 50-68% for inter-specific and 68-100% for intra-specific comparisons. The average of genetic similarity based on ITS-PCR patterns digested with *Cfo*I was approximately 50%. Dendrogram of the ITS-PCR patterns digested with *Cfo*I separated the isolates of *Cladosporium* spp. into two main clusters. The first main cluster included all *Cladosporium cladosporioides* isolates at the genetic similarity (GS = 76 %). Isolates 2, 3 and 4 showed genetic similarity (GS = 100%) (Fig. 1).

Internal Transcribed Spacer (ITS) PCR patterns of 23 *Cladosporium* spp. isolates digested with *Msp*I: The dendrogram constructed with ITS-PCR digested with *Msp*I revealed that all isolates of *C. cladosporioides* and most of the *C. sphaerospermum* can be grouped into a major cluster delimited from other *Cladosporium* spp. This is in good agreement with the classification by morphological characteristics (Fig. 2).

Internal Transcribed Spacer (ITS) PCR patterns of 23 *Cladosporium* spp. isolates digested with *Rsa*I: The genetic similarity between *Cladosporium* spp., isolates

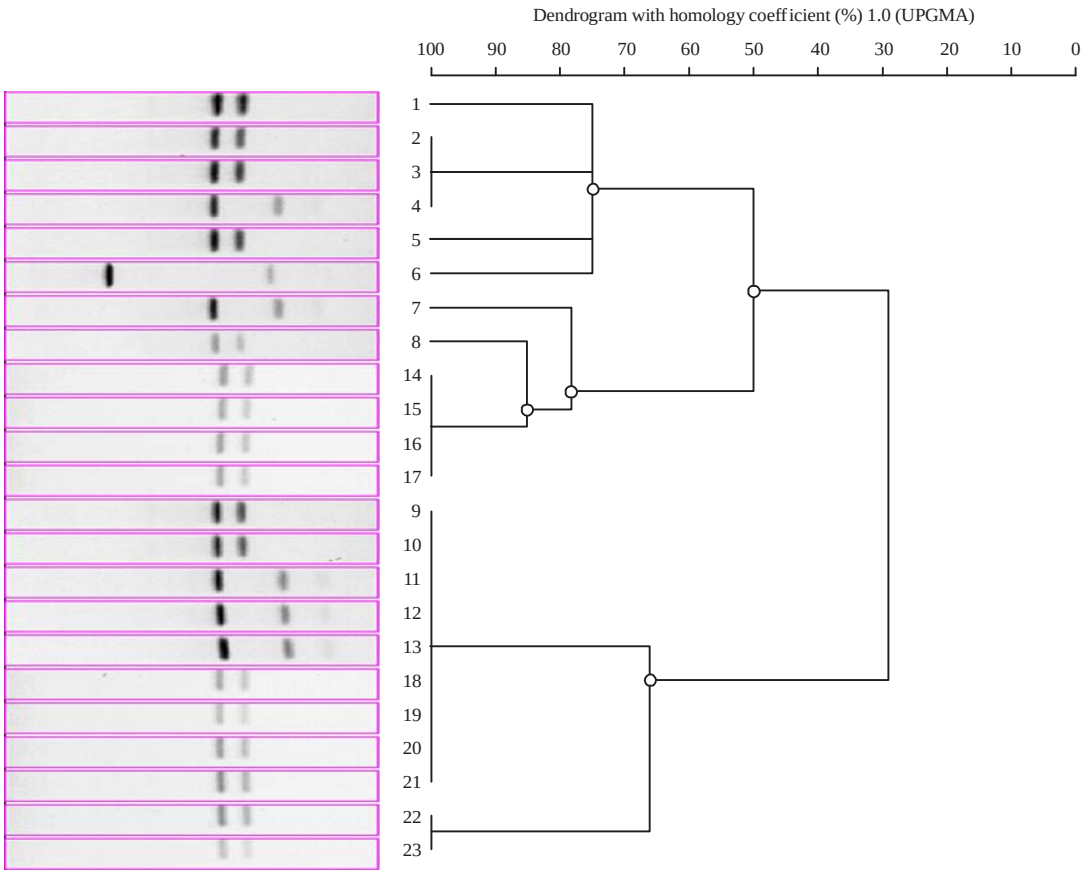

Fig. 1: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized ITS4/ITS5-PCR and digested with *Cfo*I with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

ranged from 20-42% for inter-specific and 42-100% for intra-specific comparisons. The application of UPGMA clustering produced two large clusters within the population with a branched-off at genetic similarity of GS = 20%, each consisting of several subclusters (phenons). Dendrogram of the ITS-PCR patterns digested with *Rsa*I separated the isolates of *Cladosporium* spp., into two main clusters (Fig. 3).

Molecular characterization and genetic diversity of *Cladosporium* species as revealed by microsatellite-primed PCR (MP-PCR)

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(AG)_8C$ primer: The $(AG)_8C$-primed PCR were normalized and analyzed using the BandMap software. This program calculates the Genetic Similarities (GS) and differences between each

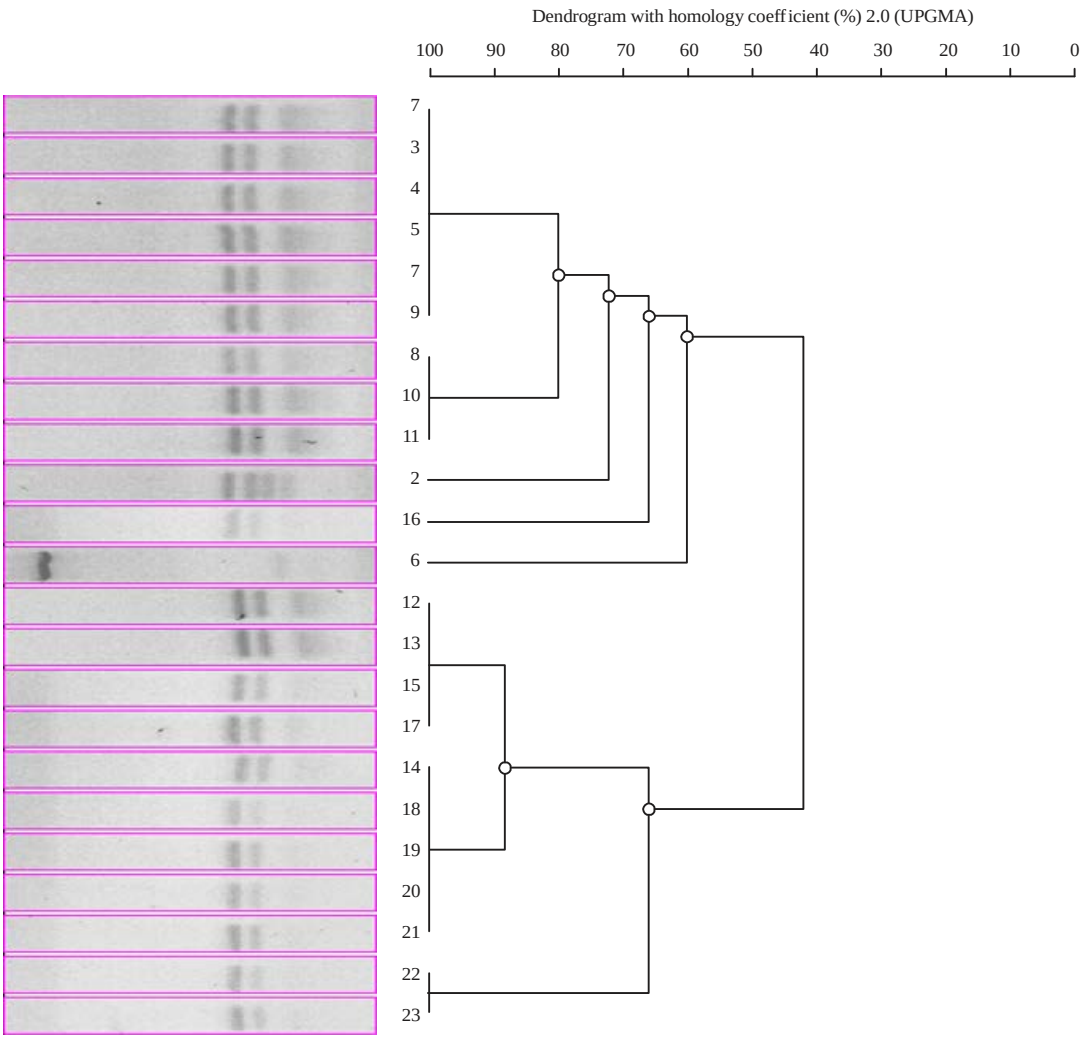

Fig. 2: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized ITS4/ITS5-PCR and digested with *Msp*I with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

MP-PCR patterns, with the Pearson's product moment correlation coefficient (r) between samples to construct a matrix. The samples were then clustered using the unweighted pair group method of arithmetic average (UPGMA), which resulted in a dendrogram. The dendrogram showing the genetic relationship among the Cladosporium strains based on the total number of amplified MP-PCR fragments is presented in Fig. 4. The genetic similarity between Cladosporium isolates ranged from 10-14% for inter-specific and 80-100% for intra-specific comparisons. The application of UPGMA clustering produced two large clusters within the population with a branched-off at genetic similarity of GS = 14%, each consisting of several subclusters (phenons).

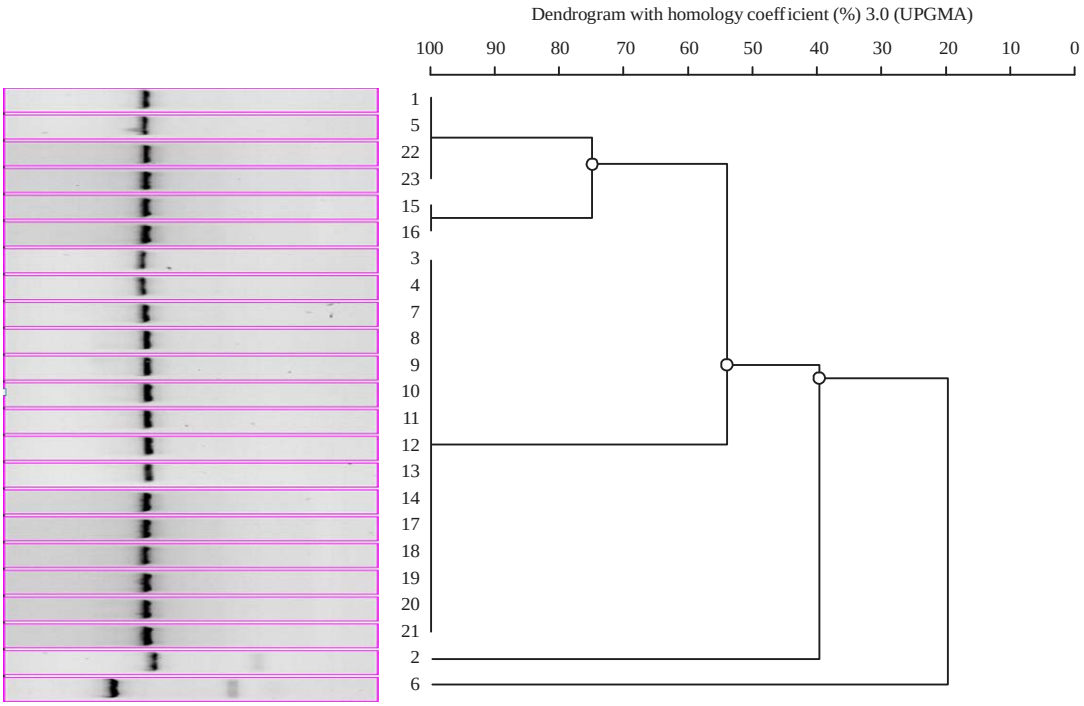

Fig. 3: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized ITS4/ITS5-PCR and digested with *Rsa*I with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. spherospermum* Penzig CBS 114326, 7: *C. spherospermum* Penzig UMC 4433, 8: *C. spherospermum* Penzig UMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(AGG)_5$ primer: All 23 isolates of *Cladosporium* spp., used in this study were typed by PCR amplification with primer $(AGG)_5$, producing a fingerprinting profile. Under the conditions stipulated, MP-PCR using the proposed primer combinations produced an average of 6 or 18 DNA fragments per isolate. Based on the cluster analysis of the genetic similarities using UPGMA clustering, two main clusters were defined (Fig. 5). Between *Cladosporium* spp., similarities ranged from 25-28% for inter-specific comparisons. The genetic similarities among all taxa ranged from 28-100% for intra-specific comparisons. The differentiation power of $(AGG)_5$ primer profiling for identification of *Cladosporium* spp., isolates was relatively poor.

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(CAG)_5$ primer: Figure 6 shows the results of cluster analysis of the MP-PCR profiles. It is noteworthy that this technique yielded 6-13 isolate-specific patterns. The UPGMA analysis of the $(CAG)_5$ data separated the *Cladosporium* spp., isolates into two main groups, each of which shared about 20% similarity. Between *Cladosporium* spp., isolates, similarities ranged from 22-28% for inter-specific and 30-100% for intra-specific comparisons. The two *Cladosporium herbarum* isolates 14 and 16 share high genetic similarity value (100%). The differentiation power of $(CAG)_5$ primer profiling for identification of *Cladosporium* spp., isolates was relatively poor.

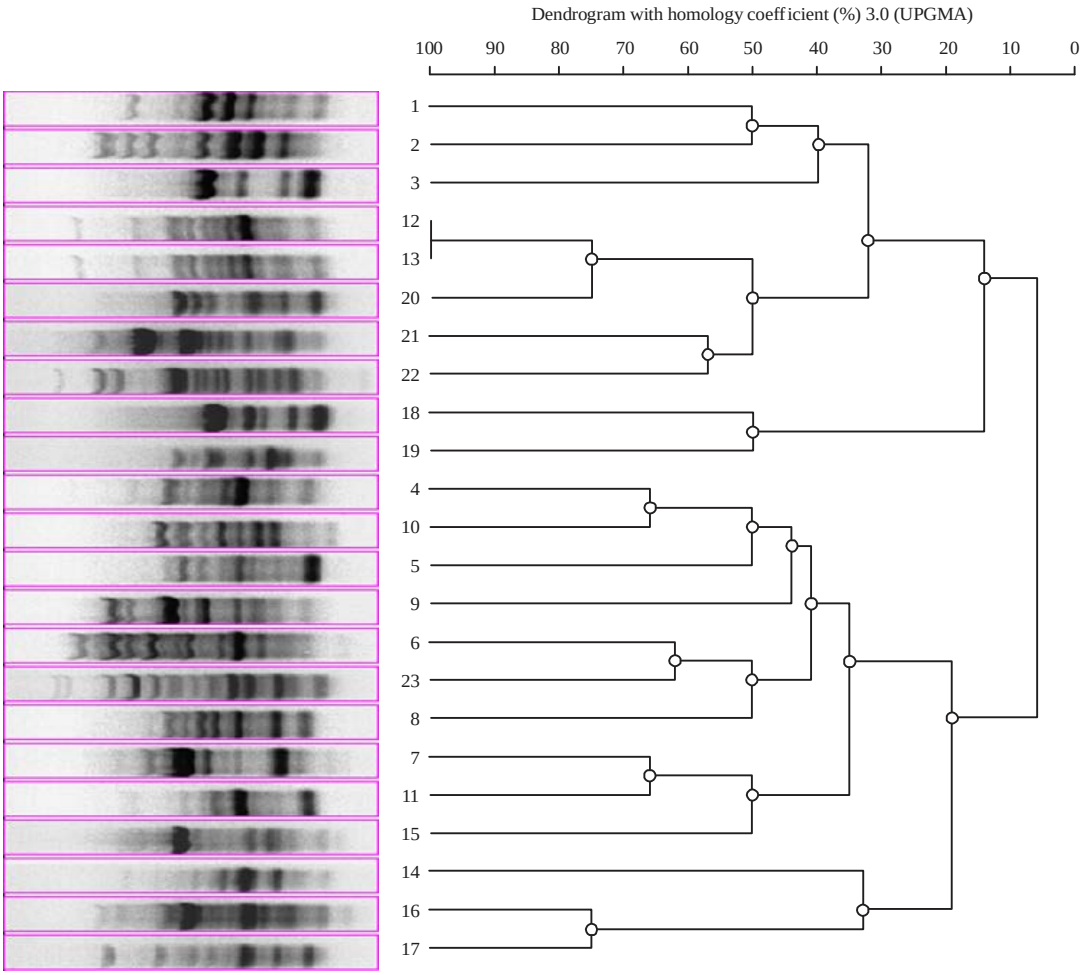

Fig. 4: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(AG)_8$C-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4 440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(GACA)_4$ primer: Based on genetic similarity values (GS), UPGMA cluster analysis was conducted to graphically display grouping between isolates and species. The clustering resulted in a single tree with no ambiguity (ties) detected in the course of linking closest pairs of isolates. Between *Cladosporium* spp., isolates, similarities ranged from 40-58% for inter-specific and 58-78% for intra-specific comparisons. Isolates from six *Cladosporium* species could be separated into two main clusters (Fig. 7). Dendrogram of the $(GACA)_4$ primer did not provide clear-cut differentiating features between *Cladosporium* spp., isolates.

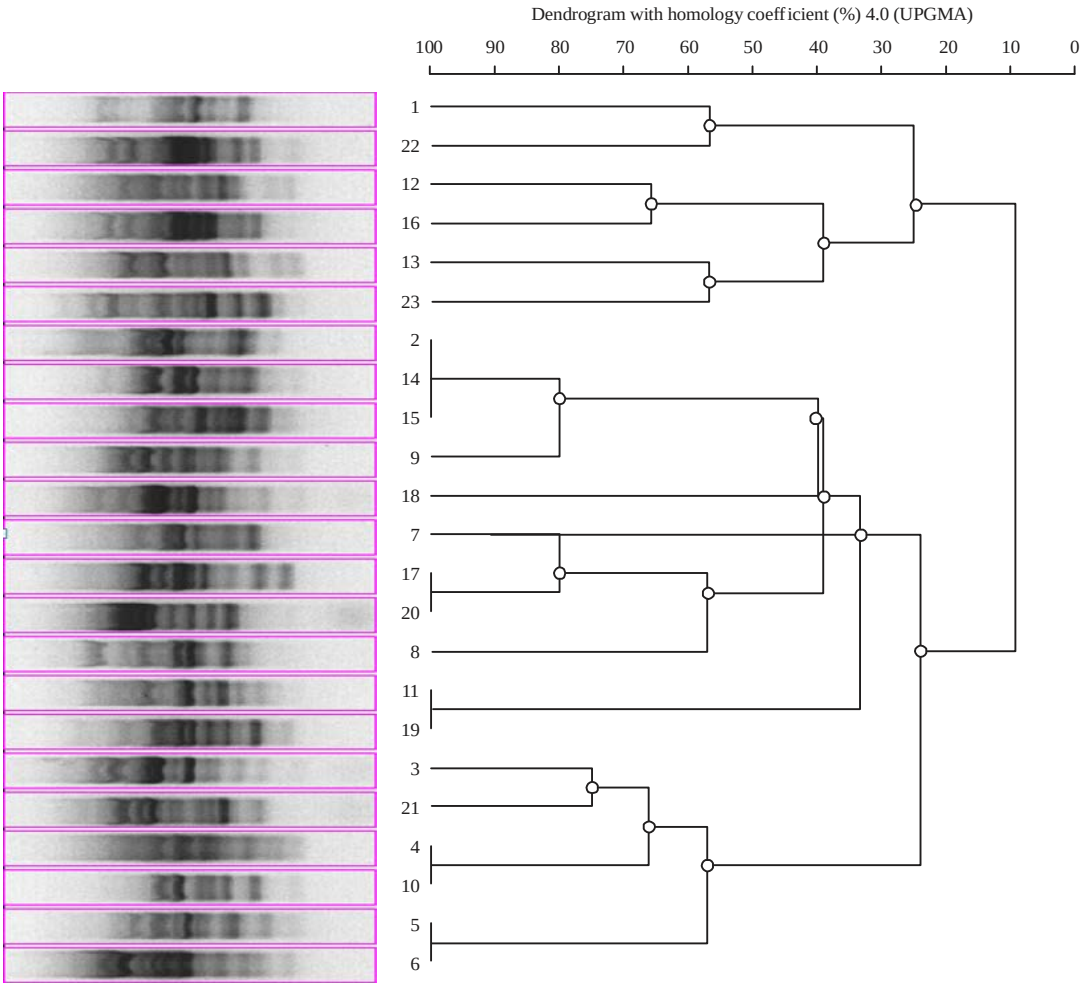

Fig. 5: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(AGG)_5$-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(GTG)_5$ primer: The phenogram obtained by cluster analysis of the $(GTG)_5$-PCR data revealed a moderate level of genetic variability between 23 geographically diverse isolates of *Cladosporium* spp. between *Cladosporium* spp., isolates, similarities ranged from 60-80% for inter-specific and 80-100% for intra-specific comparisons. Isolates from six *Cladosporium* species could be separated into two main clusters (Fig. 8). Some of the *Cladosporium* spp., isolates that had been identified as a same species showed a tendency to cluster.

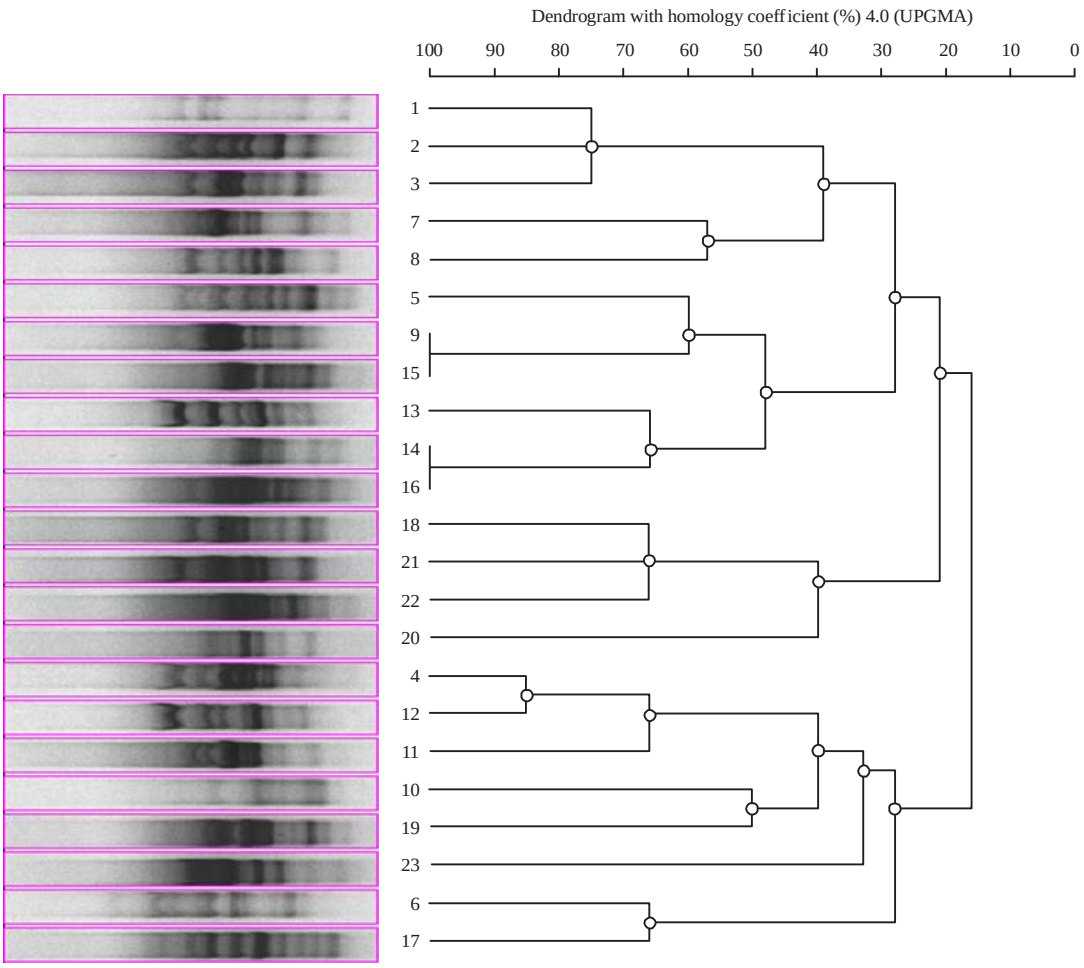

Fig. 6: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(CAG)_5$-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with $(GTGC)_4$ primer: The $(GTGC)_4$ banding patterns obtained by agarose gel were concatenated and analyzed as a composite by BandMap software. The normalization setting used were as follows: A resolution of 500 points, a smoothing factor of 3 and background subtraction by the rolling-disk method with intensity setting of 12 and the patterns were clustered by unweighted pair group method of arithmetic average. By UPGMA, all the $(GTGC)_4$-PCR markers were used to estimate the genetic relatedness among *Cladosporium* spp., isolates. The obtained dendrogram (Fig. 9) depicts that all isolates were separated from each other into two distinct groups (similarity are above 40%). The genetic similarity between *Cladosporium* spp., isolates ranged from 40-48% for inter-specific and 50-100% for intra-specific comparisons. The two *Cladosporium herbarum* isolates 18 and 19 share high genetic similarity value (100%).

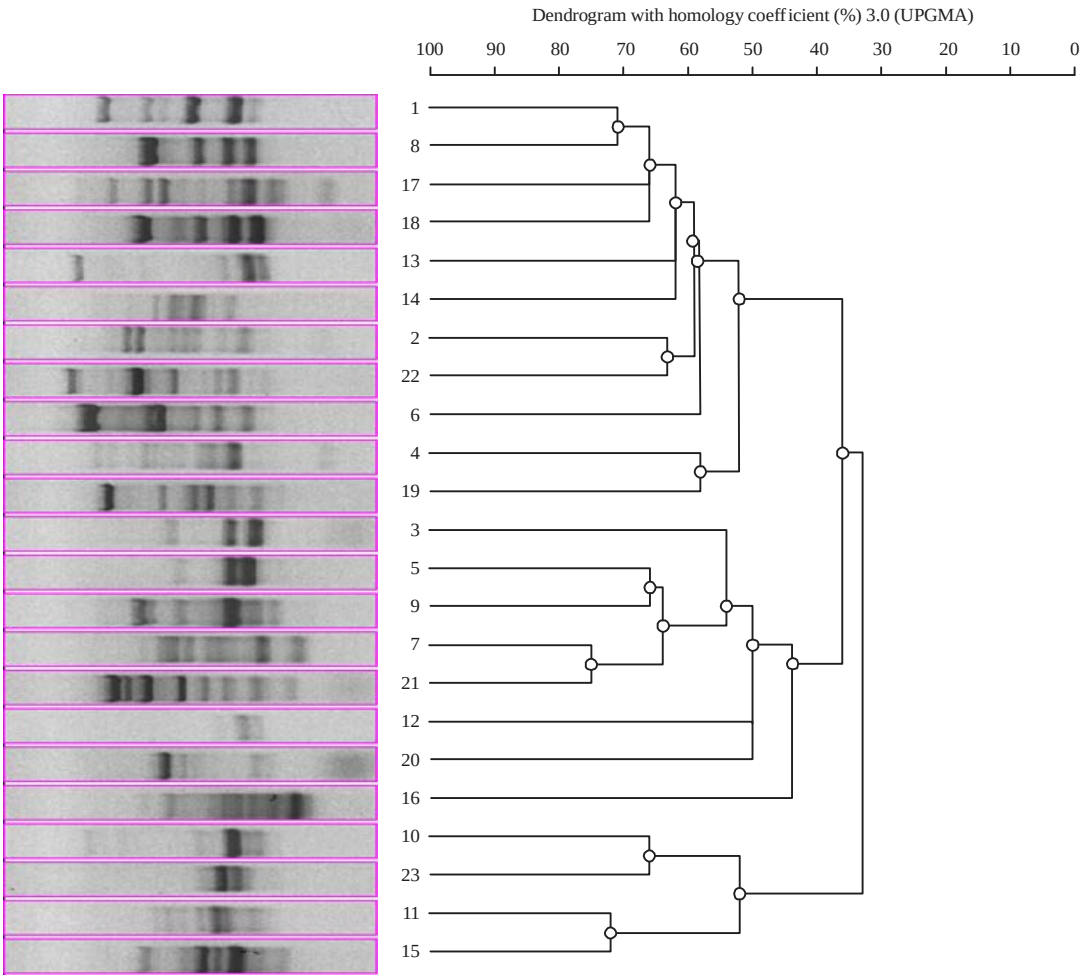

Fig. 7: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(GACA)_4$-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with T3B primer: Two major groups were observed in the dendogram (Fig. 10), which was divided into four subgroups. The average between-group genetic similarity was observed to be 50% between all *Cladosporium* spp. groups. Between *Cladosporium* spp., similarities ranged from 50-52% for inter-specific comparisons. The genetic similarities among all taxa ranged from 52-95% for intra-specific comparisons.

Microsatellite-primed PCR (MP-PCR) patterns of 23 Cladosporium isolates obtained with M13 primer: An UPGMA dendrogram produced by using BandMap software analysis, between all studied individuals is shown in Fig. 11.

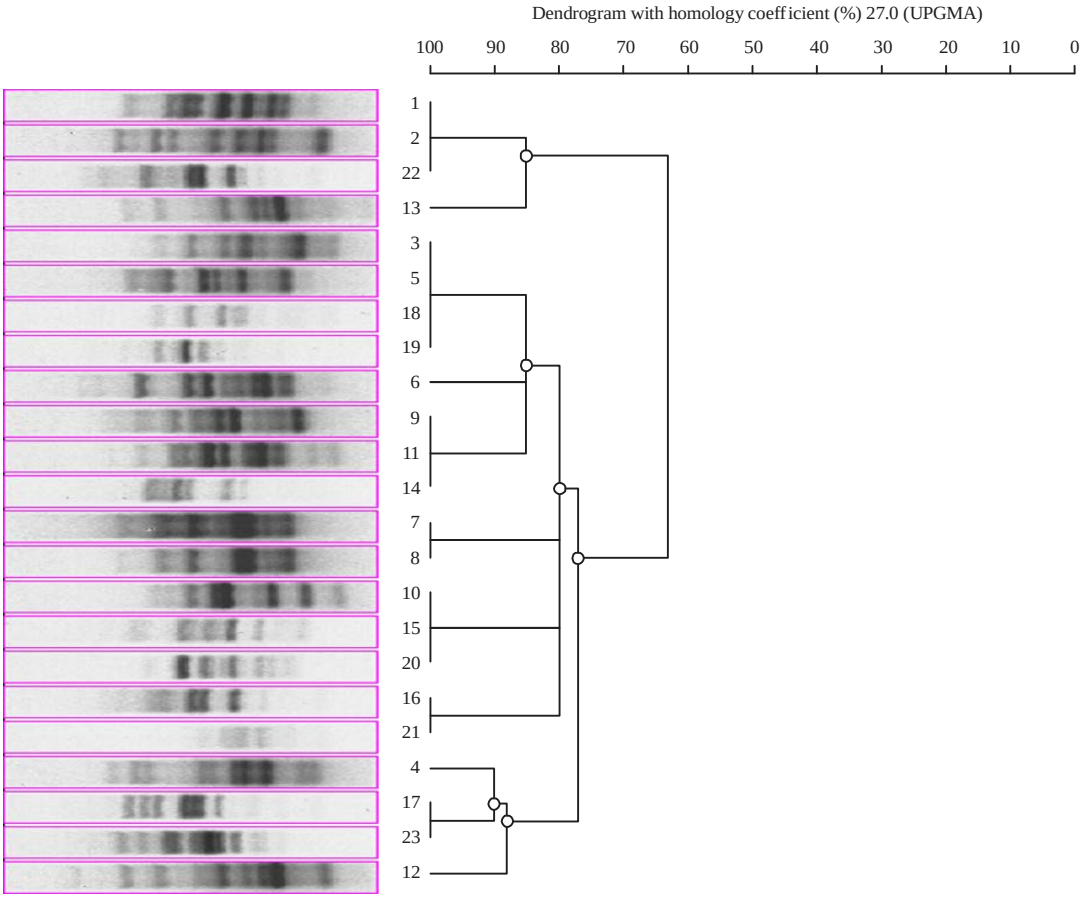

Fig. 8: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

The tree obtained indicated that the populations of *Cladosporium* spp., formed a distinct group and genetic similarity between these isolates was high (48%). There were no clear-cut kinships between clustering in the M13 dendrogram and morphological characterization of *Cladosporium* spp.

DISCUSSION

There has been an increasing interest in the application of Polymerase Chain Reaction (PCR) technology for the identification of pathogenic fungi. This technique offers the advantage of reducing or eliminating the need for lengthy culturing and difficult morphological identification procedures[12]. The DNA-based techniques provide practical markers for molecular typing of a range of fungal species. These methods are commonly used as tools in fungal taxonomy, allowing the discrimination of isolates from intrageneric to strain levels[13]. The PCR-based genomic fingerprinting is a good alternative to methods that rely on specifically targeted primers. These techniques,

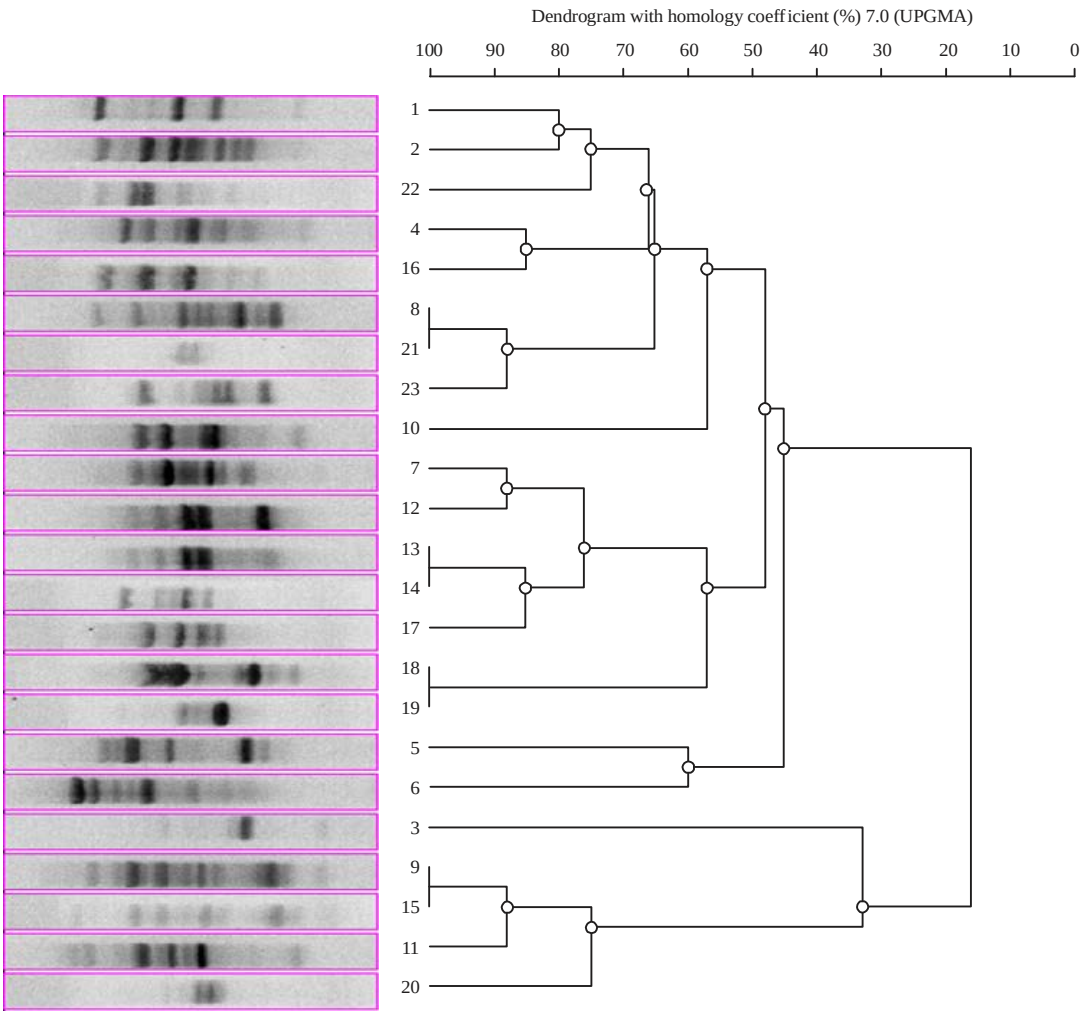

Fig. 9: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized $(GTGC)_4$-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries A U MC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link C BS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

which analyze the whole genome, have been shown to be relatively robust and discriminatory[14].

Nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2) have been used successfully to analyze intraspecific and interspecific relationships in various fungi[15]. The anamorph genus Cladosporium is one of the most widespread and prevalent of all fungal genera[16]. It lacks morphological structures that would firmly place species in an evolutionary context. Molecular data are therefore important to reveal phylogenetic relationships within this genus. In an earlier study reported by Curtis *et al.*[17], partial rDNA sequences of several independent isolates of *C. fulvum* (syns. *Fulvia fulva*, *Mycovellosiella fulva*) and single isolates of *C. herbarum*, *C. oxysporum*, *C. cladosporioides* and *C. sphaerospermum* indicated that these species together might form a monophyletic clade.

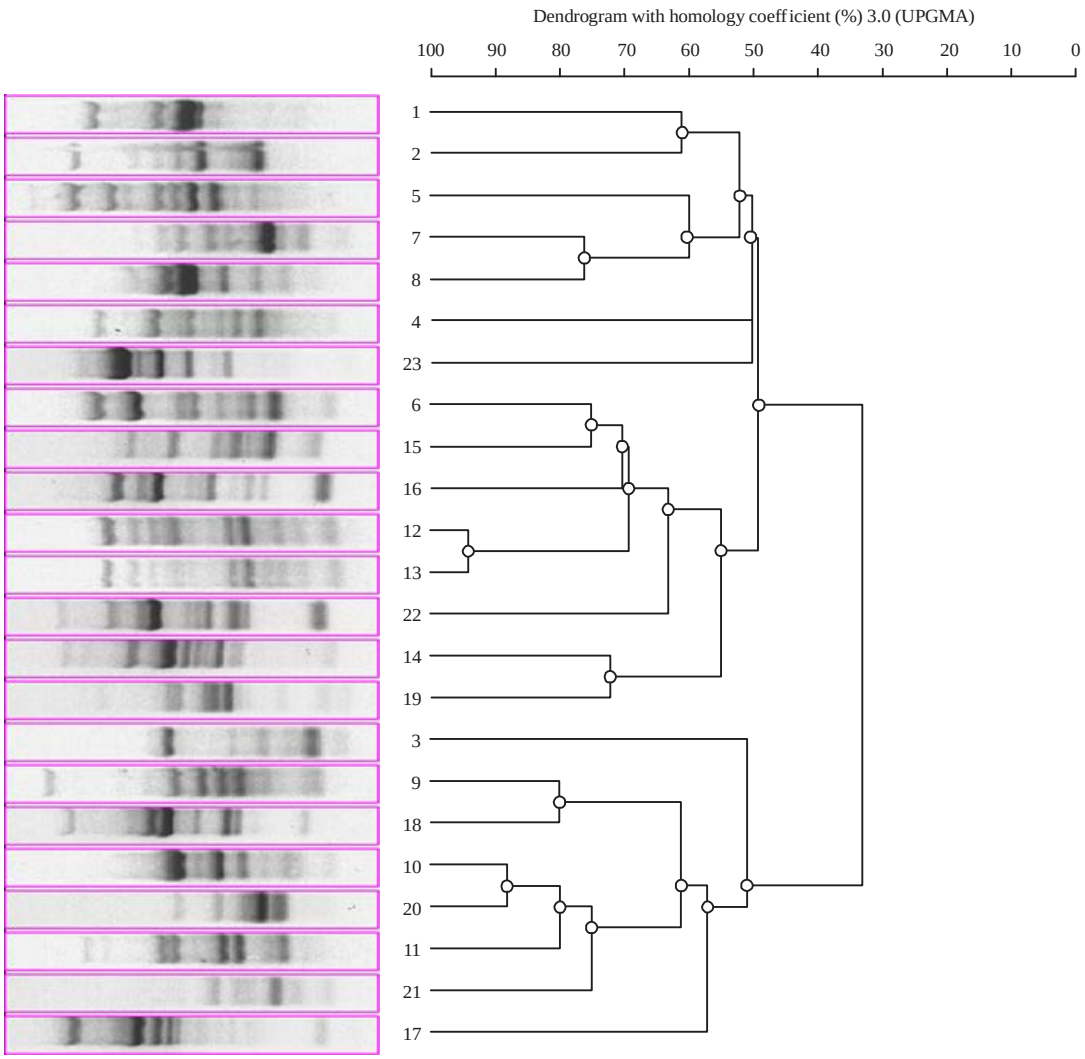

Fig. 10: Dendrogram of 23 *Cladosporium* isolates was constructed after cluster analysis of the digitized T3B-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

In the present study, the Internal Transcribed Spacer (ITS) was used to compare the ITS in length and restriction patterns. The Internal Transcribed Spacer (ITS) was amplified using PCR combining primers ITS4 and ITS5. The PCR products were digested with three restriction enzymes and separated by agarose electrophoresis. Restriction patterns generated by *Cfo*I and *Msp*I and *Rsa*I were unique for most species assayed. Clear results were obtained by using ITS-PCR in the present study. The results were consistent with those based on biological characteristics and morphological features. The ITS-PCR fingerprinting methods presented here led to clear differentiation of the isolates at the species

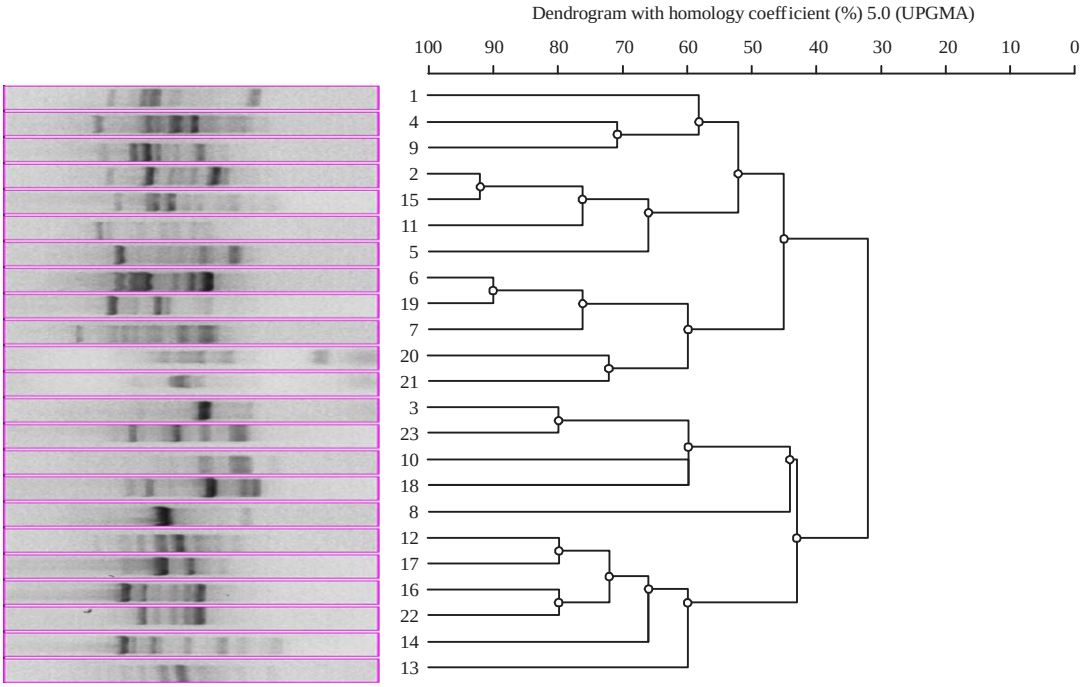

Fig. 11: Dendrogram of 23 Cladosporium isolates was constructed after cluster analysis of the digitized M13-PCR fingerprints with the unweighted pair group method with arithmetic averages (UPGMA), 1: *C. cladosporioides* (Fresen.) de Vries CBS 131.29, 2: *C. cladosporioides* (Fresen.) de Vries AUMC 4432, 3: *C. cladosporioides* (Fresen.) de Vries AUMC 4439, 4: *C. cladosporioides* (Fresen.) de Vries AUMC 4445, 5: *C. cladosporioides* (Fresen.) de Vries AUMC 4450, 6: *C. sphaerospermum* Penzig CBS 114326, 7: *C. sphaerospermum* Penzig AUMC 4433, 8: *C. sphaerospermum* Penzig AUMC 4435, 9: *C. sphaerospermum* Penzig AUMC 4440, 10: *C. sphaerospermum* Penzig AUMC 4441, 11: *C. sphaerospermum* Penzig AUMC 4444, 12: *C. sphaerospermum* Penzig AUMC 4448, 13: *C. sphaerospermum* Penzig AUMC 4449, 14: *C. herbarum* (Persoon) Link CBS 673.69, 15: *C. herbarum* (Persoon) Link AUMC 4434, 16: *C. herbarum* (Persoon) Link AUMC 4436, 17: *C. herbarum* (Persoon) Link AUMC 4437, 18: *C. herbarum* (Persoon) Link AUMC 4438, 19: *C. herbarum* (Persoon) Link AUMC 4442, 20: *C. macrocarpum* Preuss AUMC 4443, 21: *C. macrocarpum* Preuss AUMC 4446, 22: *C. chlamydosporis* Matsushima AUMC 4447 and 23: *C. tenuissimum* Cooke CBS 117134

level. Fingerprinting profiles generated discriminated between each of the 6 species. Cluster analysis further supported this observation and clusters corresponding to each species could be identified in the dendrograms. Our results support an earlier report that ITS molecular marker technique represents a possible method for the classification of *Cladosporium* spp.[11]. Additionally, the ITS restrictions profiles showed a great genetic similarity between *C. cladosporioides* and almost all of *C. sphaerospermum* isolates.

Microsatellites (tandem repeats of 1-5 base pairs) and minisatellites (tandem repeats of a basic motif 10-60 bp long) are ubiquitous components of eukaryotic genomes. The fingerprinting methods referred to as microsatellite-primed polymerase chain reaction (MSP-PCR) and repetitive-sequence based polymerase chain reaction (rep-PCR) are used to discriminate between fungal species[18].

Primer $(AG)_8C$ could not discriminate between the two *Cladosporium* spp., isolates 12 and 13 as they share high genetic similarity value (100%). The differentiation power of $(AGG)_5$ and $(CAG)_5$ primer profiling for identification of *Cladosporium* spp., isolates was relatively poor. Six microsatellites primers from eight primers were unable to generate visible DNA fingerprints. It is possible that these repeats are not present in the genomes of these fungi. Another possibility is that the primer annealing sites are at such a distance that amplification by Taq polymerase is impossible. Amplification experiments demonstrate that

microsatellite primer, T3B and $(GTG)_5$ are technically simple tools for assaying genetic variability in *Cladosporium* spp.

CONCLUSION

To conclude, in the present study, different primers provided varying levels of discrimination between morphological group representatives. Cluster analysis demonstrated that there is a genetically distinct variation among *Cladosporium* spp. These data confirmed the hypervariability of the PCR-based markers.

REFERENCES

1. Padovan, A.C.B., A.S.D.A. Melo and A.L. Colombo, 2013. Systematic review and new insights into the molecular characterization of the *Candida rugosa* species complex. Fungal Genet. Biol., 61: 33-41.
2. Hamarsheh, O., W. Presber, A. Al-Jawabreh, Z. Abdeen, A. Amro and G. Schonian, 2009. Molecular markers for *Phlebotomus papatasi* (Diptera: Psychodidae) and their usefulness for population genetic analysis. Trans. R. Soc. Trop. Med. Hygiene, 103: 1085-1086.
3. Lackner, M., R. Caramalho and C. Lass-Florl, 2014. Laboratory diagnosis of mucormycosis: Current status and future perspectives. Future Microbiol., 9: 683-695.
4. Schmidt, T. and J.S. Heslop-Harrison, 1996. The physical and genomic organization of microsatellites in sugar beet. Proc. Natl. Acad. Sci. USA., 93: 8761-8765.
5. Cleary, J.D. and L.P. Ranum, 2014. Repeat associated non-ATG (RAN) translation: New starts in microsatellite expansion disorders. Curr. Opin. Genet. Dev., 26: 6-15.
6. Miah, G., M.Y. Rafii, M.R. Ismail, A.B. Puteh, H.A. Rahim, K.N. Islam and M.A. Latif, 2013. A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. Int. J. Mol. Sci., 14: 22499-22528.
7. Bowles, D., 2015. Recent advances in understanding the genetic resources of sheep breeds locally-adapted to the UK uplands: Opportunities they offer for sustainable productivity. Front. Genet., Vol. 6.
8. Arcade, A., F. Anselin, P.F. Rampant, M.C. Lesage, L.E. Paques and D. Prat, 2000. Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch. Theor. Applied Genet., 100: 299-307.
9. George, B., C.M. Alam, R.V. Kumar, P. Gnanasekaran and S. Chakraborty, 2015. Potential linkage between compound microsatellites and recombination in geminiviruses: Evidence from comparative analysis. Virology, 482: 41-50.
10. Abd-Elsalam, K.A., A. Asran-Amal and A.M.A. El-Samawaty, 2007. Isolation of high-quality DNA from cotton and its fungal pathogens[Isolierung hochqualitativer DNA aus Baumwolle und ihren pilzlichen Krankheitserregern]. J. Plant Dis. Prot., 114: 113-116.
11. Troche, C.A.L., 1997. PCR-based DNA fingerprinting of seven *Cladosporium* species using the internal transcribed spacer from nuclear ribosomal DNA. M.S. Thesis, University of Puerto Rico, Mayaguez, Puerto Rico.
12. Alves, A., A.J.L. Phillips, I. Henriques and A. Correia, 2005. Evaluation of amplified ribosomal DNA restriction analysis as a method for the identification of *Botryosphaeria* species. FEMS Microbiol. Lett., 245: 221-229.
13. Soll, D.R., 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin. Microbiol. Rev., 13: 332-370.
14. Olive, D.M. and P. Bean, 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol., 37: 1661-1669.
15. Chase, M.W., N. Salamin, M. Wilkinson, J.M. Dunwell, R.P. Kesanakurthi, N. Haidar and V. Savolainen, 2005. Land plants and DNA barcodes: Short-term and long-term goals. Philos. Trans. R. Soc. Lond. B Biol. Sci., 360: 1889-1895.
16. David, J.C., 1997. A Contribution to the Systematics of Cladosporium: Revision of the Fungi Previously Referred to Heterosporium. CAB International, Wallingford, Oxon, UK., ISBN: 9780851989402, Pages: 157.
17. Curtis, M.D., J. Gore and R.P. Oliver, 1994. The phylogeny of the tomato leaf mould fungus *Cladosporium fulvum* syn. *Fulvia fulva* by analysis of rDNA sequences. Curr. Gen., 25: 318-322.
18. Alves, A., A.J.L. Phillips, I. Henriques and A. Correia, 2007. Rapid differentiation of species of Botryosphaeriaceae by PCR fingerprinting. Res. Microbiol., 158: 112-121.