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Morphological and Molecular Study of Different *Penicillium* Species

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Abstract: *Penicillium* is a genus of ascomycetous fungi of major importance in the environment, food and drug production. It produces penicillin, a molecule that is used as an antibiotic, this kills or stops the growth of certain kinds of bacteria inside the body. In the present investigation morphological characterization of Penicillium species was carried out in two different media (PDA and Czapek Dox). Morphological characteristics of microbes may be influence by environmental factor and any mutations at the genome can't be investigated by morphological marker so here one additional step (molecular markers reveal characterization) has been taken to overcome this type of problem to characterize some *Penicillium* species. The polymorphism at the molecular level was studied by random amplified polymorphic DNA (RAPD) marker technique and variation in the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). In our study all selected 20 random primers showed polymorphism and generated total 252 RAPD fragments of which 83.73% were polymorphic. The number of amplification products produced by each primer were varied from 4-16 with an average of 10.55. The sizes of amplified fragments were ranged from 218-2939 bp. Pair wise genetic similarity estimated the range from 0.20-0.80 Jaccords coefficient. All selected *Penicillium* species were grouped into four major clusters. *Penicillium purpurogenum* and *Penicillium notatum* was closest species in our study and as expected not higher but moderate level of similarity was found among all selected *Penicillium* species.

Key words: Fungal genomic DNA · Genetic variation · Morphological marker · ITS · RAPD markers

INTRODUCTION

Penicillium is worldwide known for production of secondary metabolites and extra cellular enzymes of commercial value [1, 2]. This enables the mycologists to be confident they are working with exactly the same species or even strains of that species [3]. It can be done by morphological, biochemical and molecular markers reveal characterization. The physical constitution of an organism that results from its genetic constitution and the action of the environment on the expression of the genes is termed the phenotypic diversity whereas genetic diversity refers to any variation in the nucleotides, genes, chromosomes, or whole genomes of organisms. The molecular marker reveal study is easy; it detects similarities up to nucleotide level so this method is very effective for microbial species characterization [4].

The molecular markers are a useful tool for assessing genetic similarity and resolving species identities. Among the molecular markers, RAPD markers typically have high overall variability and so can be useful for microbial characterization among different species [5-7]. RAPD markers have been widely used for assessing genetic diversity, genome mapping and molecular diagnostics of many fungal species. RAPD requires no prior sequence information for the fingerprinting of any genomic DNA and it has been used extensively for estimating genetic variations at the population level and among closely related species. Unlike the morphological and biochemical markers which may be affected by environmental factors, DNA markers depict genome sequence composition, thus enabling the detection of differences in the genetic information carried by the difference species and not affected by environmental factors [8]. The power of the existing DNA techniques to genetically define populations offers an attractive possibility toward characterization of useful species.

The genetic diversity of many *Penicillium* species are also being studied by random amplified polymorphic DNA (RAPD) as well as by sequencing and restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region of ribosomal DNA

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(rDNA). Regions of ribosomal DNA (rDNA) also have been used in phylogenetic studies of fungal genomes [9-11]. These regions are highly conserved and can easily be investigated using PCR amplification. Out of the various regions of rDNA, the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the nuclear rDNA repeat units have been reported to evolve fast and may vary among species within a genus or among populations [7] and hence can be used for phylogenetic study.

MATERIALS AND METHODS

Collection of Samples and Maintenance of Pure Culture: Fungal samples was taken and the present research entitled morphological and molecular characterization of *Penicillium* species was carried out at the School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India. The pure culture was maintained on PDA and Czapek Dox media (Hi media, Laboratories Ltd. Bombay, India) at 25±1°C and for morphological study mycelia was prepared by simple inoculation of sample from pure culture into PDA broth and semi solid Czapek Dox broth into 100 ml conical flasks and were incubated at 25±1°C for 7 days.

Morphological Characterization: Morphological and molecular characterization of *Penicillium* species was carried out on the basis of fungal morphology (macroscopic and microscopic) and molecular markers (RAPD and ITS) reveals characterization.

Macroscopic and Microscopic Study: Macroscopic study was done by studying growth rate, color texture and topography of colony using two standard media namely PDA and Czapek Dox. Microscopic study of different *Penicillium* species was done by preparing slide mount with lacto phenol cotton blue stain and observed under light microscope.

Fungal Mycelium Preparation and DNA Isolation: Fungal mycelium was prepared from pure culture using 50 ml of PDA broth in 100 ml conical flasks and was incubated at 25±1°C for 7 days. Mycelia from 50 ml broth were harvested by filtration through Whatman sterile filter paper and the fungal genomic DNA was isolated following the modified method of [12]. DNA concentration and purity were estimated by measurement of an optical density (OD) at wavelength 260 nm and 280 nm in spectrophotometer (Shimadzu U160A, Japan). DNA integrity was also checked by 0.8% agarose gel-electrophoresis.

Polymerase Chain Reaction and RAPD Program: Standard arbitrary 10-mer oligonucleotides (Operon Technologies Inc.; USA) were used for RAPD analysis using the method of [5]. Amplification was performed in a 25 µl reaction volume containing, Taq polymerase 10 x assay buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.01% gelatin), 0.25 mM of each dNTP, 0.6 units of *Taq* polymerase (Bangalore Genei Pvt Ltd, Banglore, India), 0.2 mM of random primer and 50 ng of DNA. Amplification was performed using Eppendorf Master Cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg, Germany), programmed for initial denaturation at 94 °C for 4 min and 36 cycles of 94°C for 1 min, 32°C for 1 min and 72°C for 1 min. The amplification was completed with a 10 min final extension at 72°C. An amplified product was resolved in 1.4% agarose gel stained with ethidium bromide (0.5 mg/ml) through electrophoresis at 60 V for 150 min, using 1X TAE buffer and photographed under UV light. 1 kb and 100bp DNA ladder served as the standard molecular weight marker regularly at left and right side. Internal transcribed spacer (ITS) region was amplified using the universal primers ITS 1 as a forward and ITS 4 as a reverse primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTTATTGATATG 3') were based on conserved 18s and 28s coding regions of the nuclear rDNA [5]. The amplification was performed in 25 µl reaction volume as described earlier. Eppendorf Master Cycler gradient was programmed for initial denaturation at 94°C for 4 min and 35 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, the amplification was completed with a final extension at 72°C for 10 min.

RESULTS

Morphological Characterization: In the present investigation total 12 *Penicillium* species namely *Penicillium citrinum, P. frequentans, P. funiculosum, P. notatum, P. oxalicum, P. purpurogenum, P. rubrum, P. rugulosum, P. variabile, P. multicolor*, *P. chrysogenum* and *P. lilacinum* was used for the morphological, RAPD and ITS reveal molecular characterization.

Macroscopic and Microscopic Characters: Macroscopic feature of *Penicillium citrinum* on Petri dish containing PDA media showed rapid growth, dark green color, granular powdery colony and the back side of colony was pale yellow in color, whereas on Czapek Dox media this species showed moderate growth, greenish orange color in granular form and the back side of the colony was yellow orange in color. *Penicillium frequentans* showed

rapid growth, green color with yellowish border and valvate appearance and the back side of colony was reddish in color with yellowish border on PDA media, whereas on Czapek Dox media this species showed rapid growth, white color with velvet appearance and the back side of the colony was greenish cream in color. *Penicillium funiculosum* showed rapid growth, white color with some brown rough appearance in center of colony and the back side of colony was creamy with reddish in color on PDA media, whereas on Czapek Dox media this species showed moderate growth, white colony with yellow, cottony, velvety colony and the back side of colony was cream in color with reddish and yellow spots. *Penicillium notatum* showed rapid growth, olive green in color, radially sulcate to plicate, margins and the back side of colony was off white in color on PDA media (Fig. 1 A, B) whereas on Czapek Dox media this species showed moderate growth, white colony and the back side of colony was colorless or dull white in color (Fig.1 A1, B1). *Penicillium oxalicum* showed rapid growth, dark green color, powdery, compact and the back side of colony was yellowish cream in color on PDA media, whereas on Czapek Dox media this species showed moderate growth, white lightly floccose in central and the back side of colony was yellow brown in color. *Penicillium purpurogenum* showed rapid growth, olive green, yellow margin, velvety colony, with granular appearance and the back side of colony was yellowish cream on PDA media (Fig. 1 D, E), whereas on Czapek Dox media this species showed rapid growth, lemon yellow border with central green powdery appearance while the back side of colony was yellow orange later it had becomes reddish in color (Fig. 1 D1, E1). *Penicillium rugulosum* showed rapid growth, dark green and powdery appearance and the back side of colony was yellowish in color on PDA media, whereas on Czapek Dox media this species showed rapid growth, white cream and after maturity some brown granular like appearance occurred while the back side of colony was pale yellow in color. *Penicillium variabile* showed moderate growth, colony was initially off white in color and after maturity it had becomes gray green and powdery and the back side of colony was off white in color on PDA media, whereas on Czapek Dox media this species showed moderate growth, initially showed light pink cottony colony after maturity it had become light green in color while the back side of colony was cream in color with yellowish border. *Penicillium rubrum* showed rapid growth, initially the colony was light yellow after maturity it had become reddish with some cottony texture and central region was velvety while the back side of colony was reddish in color on PDA media, whereas on Czapek Dox media this species showed moderate growth, dark red with whitish in color while the back side of colony was reddish in color. *Penicillium chrysogenum* showed moderate growth, green color center of colony was white and the back side of colony was yellow in color on PDA media whereas on Czapek Dox media this species showed moderate growth with yellow front and back view. *Penicillium lilacinum* showed rapid growth, the colony was white while the back side of colony was off white on PDA media whereas on Czapek Dox media this species showed moderate growth with dark yellow color and the back side of colony was cottony light yellow in color. *Penicillium multicolor* showed rapid growth, dark green

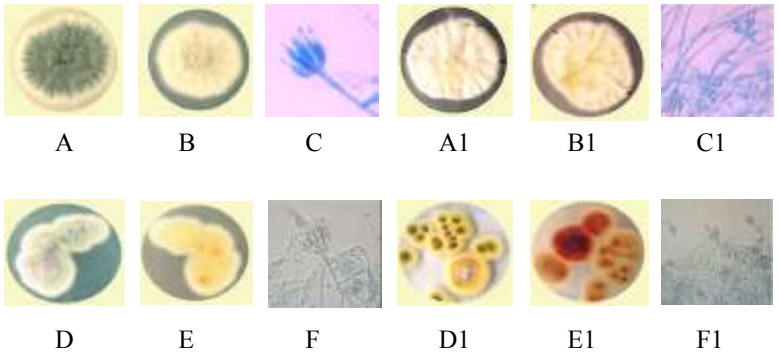

Fig. 1: A Front view of *Penicillium notatum* on PDA media, B Back view of *Penicillium notatum* on PDA media, C Microscopic structures of *Penicillium notatum* on PDA media (10 X 100X), A1Front view of *Penicillium notatum* on Czapek Dox media media, B1 Back view of *Penicillium notatum* on Czapek Dox media, C1 Microscopic structure of *Penicillium notatum* on PDA media (10 x 40X), D Front view of *Penicillium purpurogenum* on PDA media, E Back view of *Penicillium purpurogenum* on PDA media, F Microscopic structure of *Penicillium purpurogenum* on PDA media (10 x 100X), D1 Front view of *Penicillium purpurogenum* on Czepek Dox media, E1 Back view of *Penicillium purpurogenum* on Czepek Dox media, F1 Microscopic structure of *Penicillium purpurogenum* on PDA media (10 x 40X).

Table1: Morphological characteristics growth, front view, back view and characteristics of hyphae of different *Penicillium* species

Name of *Penicillium* species	Media PDA/Czapek Dox	Growth	Front view	Back view	Character of hyphae
P. citrinum	PDA	Rapid	Dark green	Pale yellow	Septate
	Czapek Dox	Moderate	Orange green	Orange yellow	
P. frequentans	PDA	Rapid	Green with yellow border	Red with yellow border	Septate
	Czapek Dox	Rapid	White	Greenish cream	
P. funiculosum	PDA	Rapid	White with brown appearance	Cream with red and yellow spot	Arial
	Czapek Dox	Moderate	White	Cream with red and yellow spot	
P. notatum	PDA	Rapid	Olive green	Off white	Smooth
	Czapek Dox	Moderate	White	Dull white	
P. oxalicum	PDA	Rapid	Dark green	Cream yellow	Smooth
	Czapek Dox	Moderate	Light white	Brown yellow	
P. purpurogenum	PDA	Rapid	Olive green	Cream	Arial and smooth
	Czapek Dox	Rapid	Green powdery	Orange yellow	
P. rugulosum	PDA	Rapid	Dark green	Yellow	Arial and smooth
	Czapek Dox	Rapid	White cream	Pale yellow	
P. variabile	PDA	Moderate	White	Off white	Smooth
	Czapek Dox	Moderate	Light pink	Creamy with yellow border	
P. rubrum	PDA	Rapid	Light yellow	Red	Arial and smooth
	Czapek Dox	Moderate	Dark red	Red	
P. chrysogenum	PDA	Moderate	Green	Yellow	Septate
	Czapek Dox	Moderate	Yellow	Yellow	
P. multicolor	PDA	Rapid	Dark green with yellow	Valvate orange	Septate
	Czapek Dox	Rapid	Dark red	Cottony white	
P. lilacinum	PDA	Rapid	White	Off white	Smooth and septate
	Czapek Dox	Moderate	Dark yellow	Cottony yellow	

Table 2: Random ten decamer primers used in present study, their sequence, number of polymorphic products, percent of polymorphic band and size of band produced by each primer

RAPD primers	Primer sequence	Total number of bands amplified	Number of polymorphic bands	Percentage of polymorphic bands	Size of band in base pair
OPC-01	TTCGAGCCAG	13	12	92.30	236-1013
OPC-03	GGGGGTCTTT	10	10	100	302-2236
OPC-04	CCGCATCTAC	10	10	100	313-1732
OPC-05	GATGACCGCC	10	9	90	418-2376
OPC-06	GAACGGACTC	11	11	100	273- 1353
OPC-08	TGGACCGGTG	4	4	100	485-1317
OPC-09	CTCACCGTCC	15	15	100	252-1599
OPC-12	TGTCATCCCC	7	7	100	404-1273
OPC-13	AAGCCTCGTC	14	13	92.85	321-1583
OPC-19	GTTGCCAGCC	13	13	100	338-1806
OPE-07	GAGTGCAGCC	11	10	90.90	808-2939
OPE-17	CTACTGCCGT	11	10	90.90	665-2775
OPG-02	GGCACTGAGG	16	15	93.75	246-2378
OPP-08	ACATCGCCCA	10	9	90	761-2463
OPP-12	AAGGGCGAGT	16	15	93.75	218-2827
OPY-07	AGAGCCGTCA	6	6	100	270-1750
OPY-11	AGACGATGGG	10	10	100	472-2018
OPZ-12	TCAACGGGAC	13	13	100	318-2678
OPZ-16	TCCCCATCAC	14	13	92.85	280-2580
OPZ-18	AGGGTCTGTG	9	7	77.77	325- 1790
Total		252	211		

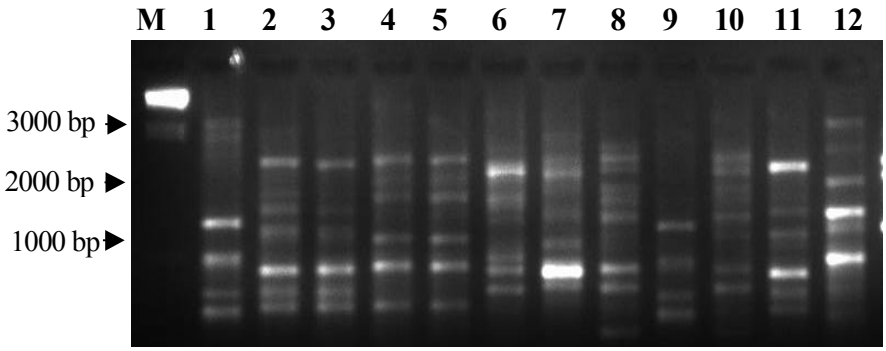

Fig. 2: RAPD banding pattern of 12 *Penicilium* species generated by random primer OPG 02. Molecular weight markers (in base pairs) are indicated on the left side (1 kb Ladder, Fermentas).

colony with light yellow and white border and the back side of colony was cottony valvate orange in color on PDA media whereas on Czapek Dox media this species showed rapid growth with dark red color and the back side of colony was cottony white in color (Table 1).

The fungal thallus typically consists of microscopic threads or filaments which branches in all direction, each of these filaments is known as hyphae. Microscopic feature of *Penicillium citrinum* showed septate hyphae, conidiophore was attached to the septate hyphae, conidia were spheraidal to subspheroidal. *Penicillium frequentans* showed septate hyphae, simple and branched conidiophores, conidia was round and unicellular. *Penicillium funiculosum* showed aerial hyphae which was sometimes pigmented and conidia was cylindrical to ellipsoidal and smooth walled. *Penicillium notatum* showed smooth hyphae typically terverticillate, conidia were commonly spheroid (Fig. 1 C, C1). *Penicillium oxalicum* showed smooth mycelium, conidia was ellipsoidal with smooth wall in long chain. *Penicillium purpurogenum* showed aerial strips long and smooth hyphae, conidia was ellipsoidal, thick wall and roughed or varicose in short irregular column (Fig. 1 F, F1). *Penicillium rugulosum* showed aerial hyphae, strips with smooth walls and conidia were ellipsoidal and smooth. *Penicillium rubrum* showed aerial mycelium; strips long smooth wall bearing biverticillate penicillin narrow and the conidia was smooth strongly. *Penicillium variable* showed smooth, much shorter hyphae, conidiophores typically biverticillate and the conidia were normally ellipsoidal, smooth or less regulose and the size of. *P. chrysogenum* showed septate hyphae and conidia were ellipsoidal. *P. liliacinum* showed smooth hyphae and conidia were ellipsoidal to fusiform. *P. multicolor* showed septate hyphae and conidia were spheraidal (Table 1).

RAPD Data Analysis: Differences in fingerprinting patterns between isolates were assessed visually only clear and reproducible bands were scored the DNA bands were scored as zero (absence) or one (presence). Data were used for similarity based analysis using the programme NTSYS-PC (version 2.02) [13]. The SIMQUAL programme was used for calculating the similarity index. A total of 20 random primer were tested (Table 2), polymorphic bands were obtained with all the 20 random primer used for amplification. The average numbers of polymorphic bands observed per primer were 10.55. The number of bands generated by each primer that produced a polymorphic banding pattern varied from 1 (OPC-08) to 15 (OPP-12) on an average, the approximate product size ranged from 218 bp to 2939 bp. One representative RAPD profile using RAPD primer (OPG-02) and banding pattern is shown in (Fig. 2). A dendrograme based on UPGMA analysis indicated that the 12 isolates formed four major clusters ABCD (Fig. 4). The similarity coefficient ranged from 0.20 to 0.80 indicating that no two isolates were 100% similar. Cluster A was further subdivided into two sub cluster which separate the *Penicillium lilacinum* from *P. citrinum* and *P. rugulosum* at coefficient value of 25% out of the 3 isolates two *P. citrinum* and *P. rugulosum* were similar with 56% similarity. Cluster B contain 4 isolates of which, *P. purpurogenum* and *P. notatum* were similar with 80% similarity. This was very closest group in our study both these two isolates were similar with *P. multicolor* with 58% similarity. Cluster C contained 3 isolates in which *P. frequentans* were separated from two isolates *P. variable* and *P. chrysogenum* with 32% similarity and this two isolates were similar with 65% similarity. Cluster D contained only two isolates *P. rubrum* and *P. oxalicum* which was similar with 63% similarity.

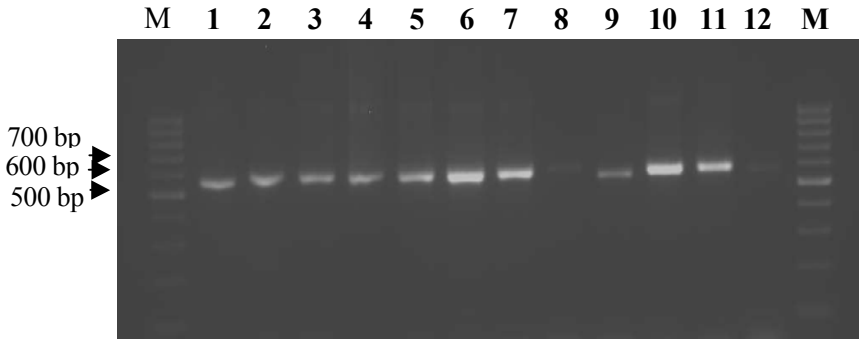

Fig. 3: Internal transcribed spacer (ITS) banding pattern of 12 *Penicillium* isolates. ITS region was amplified using the, universal primers ITS 1 as a forward (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 as a reverse primers (5' TCCTCCGCTTTATTGATATG 3'). The lane M represents 1Kb molecular size marker, on the left and right side (1Kb and 100bp, Fermentas)

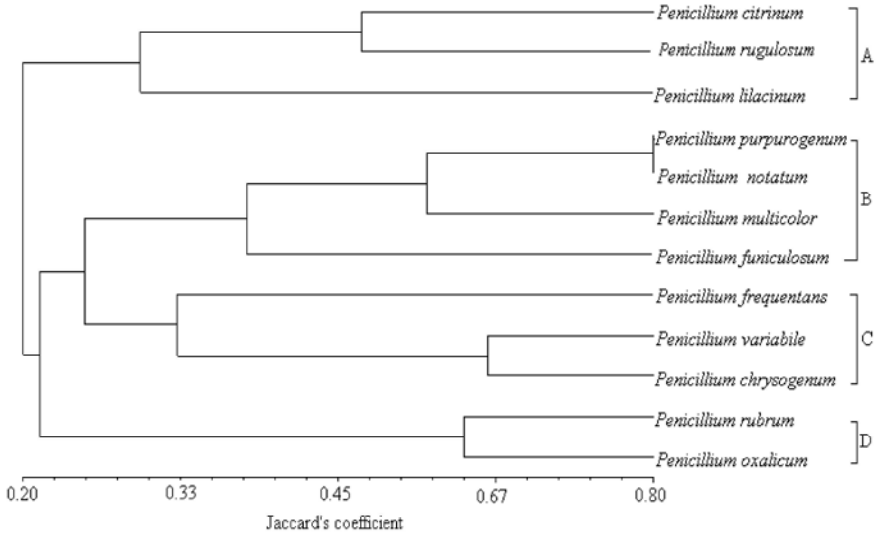

Fig. 4: UPGMA cluster analysis- based dendrogram depicting genetic relationship among different *Penicillium* species. Calculated from 211 polymorphic bands generated by 20 RAPD primers

The consensus primers ITS1 and ITS4 were used to amplify a region of the rDNA gene repeat unit. All the isolates amplified a single band of about 550 bp except two species. *Penicillium variable* showed slight increases band 565 bp in size whereas *Penicillium rugulosum* showed band of size 540 bp which was lowest in our study (Fig. 3). A recent study of *Penicillium* isolates based on banding pattern of ITS region has shown a more similarity among different species of *Penicillium*.

DISCUSSION

Different concepts have been used by mycologist to define the fungal diversity one of them is morphological study which is the classic approach where units are defined on the basis of morphological characteristics and ideally by the differences among them but this type of study is not sufficient for diversity study whereas the genetic diversity on the basis of molecular marker defeat differences among organism on the basis of size of DNA amplified which not influence by environmental factor. Variations (mutations) on nucleotides can't be studied by morphological markers while the molecular marker reveal study may overcome such type of problem therefore molecular marker reveal characterization is very effective for microbial species characterization.

In the present investigation RAPD analysis of two *Penicillium* species *Penicillium purpurogenum* and *P. notatum* showed 80% similarities while this two species

showed moderate level of similarity in morphological characteristics. *P. rubrum* and *P. oxalicum* showed genetically 63% similarity, while this two species showed similar rapid growth into PDA media, moderate growth into Czapek Dox media, the color texture was dissimilar while the hyphae was similar (smooth) in both species. *P. citrinum* and *P. rugulosum* showed genetically 56% similarities, while this two species showed similar rapid growth into PDA media, it was dissimilar in Czapek Dox media, color texture was moderately similar in both species and the hyphae was septate in *P. citrinum* whereas it was smooth in *P. rugulosum. P. chrysogenum* showed 32% similarity with *P. variable*, morphologically this species showed, similar moderate growth with green color colony on both PDA and Czapek Dox media and the hyphae of both species was dissimilar it was arial and smooth in *P. rugulosum* while it was septate in *P. chrysogenum. P. multicolor* showed 58% similarity with *P. purpurogenum* and *P. notatum* similar rapid growth and green color was observed in this three species while the hyphae was smooth in *P. purpurogenum* and *P. notatum* but it was smooth and septate in *P. multicolor. P. lilacinum* showed 25% similarity with *P. citrinum* and *P. regulosum*, similar rapid growth and green color was observed among *P. citrinum* and *P. regulosum* while the *P. lilacinum* showed white color in PDA media and dark yellow in Czapek Dox media, microscopically *P. rugulosum* and *P. lilacinum* showed smooth hyphae while the *P. chrysogenum* showed the septate hyphae, this study is similar to the study of [4].

The genetic diversity of some *Penicillium* species was also reported by random amplified polymorphic DNA [14, 15]. The molecular marker reveal characterization showed the relatively considerable level of similarities among 12 *Penicillium* species while the morphological characterization showed moderate level of similarities, similar investigation was reported by [16] in pectinase producing fungi *Penicillium expansum* and *P. graseoseum.* In our study all tested [5] primer was polymorphic, is indicating that very distinct primer can be observe for species specific when used to study different species, but identical patterns from RAPD analysis of different *Penicillium* species with 21 random primers was reported by [17]. Similar studies for molecular characterization based on RAPD markers among 10 *Penicillium* were reported by [18]. In present study high similarity was detected between *P. purpurogenum* and *P. notatum* but [16, 18] detected similarity between *P. purpurogenum* and *P. crustosum*, *P. expansum* and *P. griseoroseum*, probably due to the different number of primers utilized in every study. RAPD analysis was performed for different *Penicillium* species using 20 random primers despite the fact that in [4, 12, 18].

In this study ITS primer amplified the rDNA region of 12 *Penicillium* species and found a single monomorphic band of about 560 bp among 10 species, band variations were found only in two species. Similar investigation was performed in different *Penicillium* species and reported a monomorphic fragment of about 600 bp [15, 17, 19]. Consequently, the size of the amplified ITS region is similar to that reported for other *Penicillium* species except for *P. variable*, which contains a 1 kb ITS region as characterized by [20]. Present study investigated that the integration of different methods and techniques should lead to the identification of useful markers for the standardization of global taxonomical studies of *Penicillium* species. Our results indicated that this work has aided the development in fungal studies to make it more valuable into commercial powerhouses for the production of industrial helpful enzymes and pharmaceuticals.

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