RESEARCH ARTICLE

Genetic Diversity of Pathogenic Fungi *Aspergillus flavus* Isolates Using Random Amplified Polymorphic DNA-Polymerase Chain Reaction Technique

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ABSTRACT

Aspergillus flavus is the second most public fungi that causing disease in human and causes diseases in several important agricultural crops. The aim of this study was to investigate the genetic variety among diverse isolates of Aspergillus flavus by the technique of polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD). Five isolates were obtained from Samarra hospital and outpatient clinic and were diagnosed morphologically and microscopically. Results of RAPD profile revealed a high genetic distance (0.244) between isolates number (1 and 5), while the isolates number (2 and 5) were joined with lower genetic distance (0.090). Unique and absent bands were displayed by certain isolates and can be taken as a positive marker for isolate identification. The dendrogram of banding patterns revealed a great relationship between isolates number (2 and 5) from one side and isolates number (1 and 3) from the other side, while the isolate number 4 was separated into particular subgroup which closer to the isolate number 1 and 3.

Keywords: *Aspergillus flavus*, DNA polymorphism, Genetic diversity, RAPD.

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INTRODUCTION

Fungi are eukaryotic organisms, multicellular or unicellular shape. Commonly, they are parasitic on plants and less than are parasitic on animals and human.[1] Fungi were appeared as a main public health problem for human both in developed as well as developing countries. Nowadays, the occurrence of opportunistic fungi were significantly increased especially for the people with immunocompromised.[2,3] Some species of fungi may be causes otitis external infections like yeast and saprophytic filamentous fungi.[4] Filamentous fungi are an important fungus causing frequent infections in immunocompromised individuals due to the fungal drug resistance which used as a prophylactic in antifungal therapies.[5]

Otomycosis the chronic, acute and sub-acute inflammation of external auditory duct that causing by fungi.[6] This infection was attached with different risk factors like hot weather, bacterial infection, swimming, dusty environment, instillation of oil, ear surgery and genetic factors.[7,8] Otomycosis infection was increased due to the large number of immunocompromised patients.[9] The prevalence of otomycosis is a higher in the 21–30 years old and lower in the younger, children 10 years old, and individuals above 60 years old.[10]

The genus *Aspergillus* is one of the most common fungal species in nature, it is a main fungi involved in the cause of otomycosis in tropical and subtropical countries.[11]

Table 1: Primers and their sequences used in this study

No.	*No of primer*	*Code of primer*	*Sequence (5 to 3)*
1	P1	OPA-01	CAGGCCCTTC
2	P2	OPA-06	GGTCCCTGAC
3	P3	OPB-14	TCCGCTCTGG
4	P4	OPB-20	GGACCCTTAC
5	P5	OPC-08	TGGACCGGTG
6	P6	OPC-16	CACACTCCAG
7	P7	OPD-03	GTCGCCGTCA
8	P8	OPD-18	GAGAGCCAAC
9	P9	OPE-03	CCAGATGCAC
10	P11	OPF-05	CCGAATTCCC
11	P12	OPF-20	GGTCTAGAGG
12	P15	OPH-08	GAAACACCCC

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Table 2: The number of amplified and polymorphic bands, unique bands, absent bands, main bands, percent of polymorphism, primer efficiency and discriminatory power in *Aspergillus flavus* isolates amplified with RAPD primers.

Number	*Primer*	*Amplified bands*	*Polymorphic bands*	*Unique bands*	*Absent bands*	*Main bands*	*polymorphism %*	*Primer efficiency%*	*Primer efficiency%*
1	OPA-01	23	8	1	1	15	34.87	3.29	9.09
2	OPA-06	14	4	1	-	10	28.57	1.64	4.54
3	OPB-14	23	3	1	-	20	13.04	1.23	3.40
4	OPB-20	18	8	-	2	10	44.44	3.29	9.09
5	OPC-08	15	15	1	3	-	100	6.17	17.04
6	OPC-16	29	9	-	1	20	31.03	3.70	10.22
7	OPD-03	10	5	-	-	5	50	2.05	5.68
8	OPD-18	25	10	1	1	15	40	4.11	11.36
9	OPE-03	11	5	-	1	5	54.54	2.46	6.81
10	OPF-05	28	13	1	-	15	46.42	5.34	14.77
11	OPF-20	20	-	-	-	20	0	0	0
12	OPH-08	27	7	-	1	20	25.92	2.88	7.95
Total		243	88	6	10	155			

Table 3: Genetic similarity coefficient matrix for *A. flavus* isolates based on RAPD profile.

	1	2	3	4	5
1	0.000				
2	0.158	0.000			
3	0.160	0.183	0.000		
4	0.178	0.178	0.180	0.000	
5	0.244	0.090	0.214	0.211	0.000

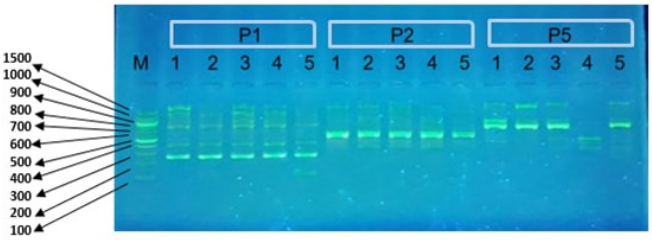

Figure 1: RAPD analysis of genomic DNA from isolates of Aspergillus flavus with the primers; OP A-01; OP A-06; OP C-08; Lane M: 100 pb ladder

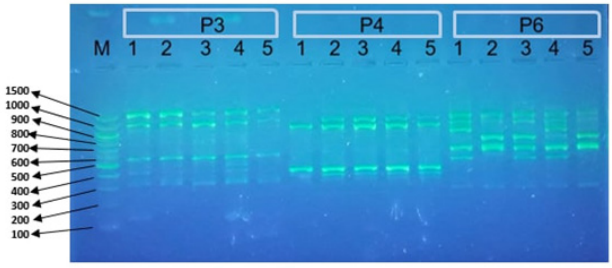

Figure 2: RAPD analysis of genomic DNA from isolates of Aspergillus flavus with the primers; OP B-14; OP B-20; OP C-16; Lane M: 100 pb ladder

Aspergillus genus was belong to filamentous fungi, grow on soil, vegetable, jellies, fruits, textile, paper, and pickles.[12] It contains more than 200 species like *A. flavus, A. niger, A. fumigatus*.[13] These species were recognized as opportunistic fungi and causing several human disease including otomycosis, endophthalmitis, keratitis, sinusitis and cutaneous infections.[14,15] *Aspergillus spp* was associated to pulmonary disease and causing invasive aspergillosis a life- threatening diseases for the patient with hematology and solid organ transplant units.[16] The most fungal infections are transfer through respiratory tract as the primary way to diffusion to the other organs of human.[17] The host was whiffed *aspergillus* conidia that grow in the lower airways and its hyphae was infest pulmonary tissues and blood vessels and spread to skin, bone, and brain and to the other organs.[18]

A. flavus the most famous species in the *Aspergillus* genus, it is a haploid filamentous fungus described by Link in (1809), found mainly in tropical and subtropical countries.[19] Humidity and atmospheric compositions are considered the most essential variables having major effect on fungus growth.[20] *A. flavus* as a saprophytic soils fungus, plays a great role in recycler the nutrient of soil propped by animal and plant wreckages.[21] The danger of *A. flavus* lies in the production of secondary metabolites including aflatoxins that considered the one of the most public mycotoxins and the great distribution and dangerous to animal and human health due to its carcinogenic besides the toxic effects, in addition, it causes diseases on several important agricultural crops such as maize, peanut, and cotton seed.[22,23] Aflatoxins B1, B2, G1, and G2 are the main four toxins among at less sixteen structurally associated toxins, *A. fluvus* produce aflatoxins B1, B2.[24]

A. flavus was appeared a great source of comparative information, and has eight chromosomes with the size of a genome of about 36 Mb also, to 13.071 as prophecy genes.[25] Methods used to distinguish aspergillus species depending on morphological and microscopic characters whicn are deficient and deceptive due to the slow growth of fungi and some characters are homogenous to several species within the

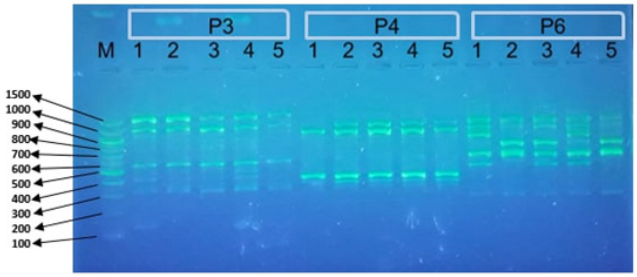

Figure 3: RAPD analysis of genomic DNA from isolates of Aspergillus flavus with the primers; OP D-03; OP D-18; OP E-03; Lane M: 100 pb ladder

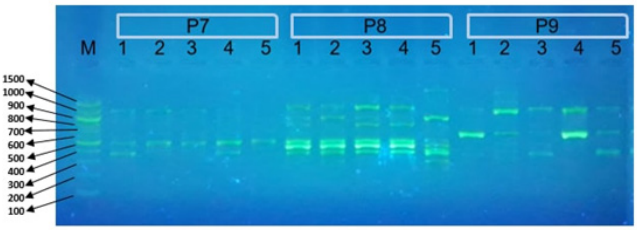

Figure 4: RAPD analysis of genomic DNA from isolates of Aspergillus flavus with the primers; OP F-05; OP F-20; OP H-08; Lane M: 100 pb ladder

genus.[26] The evolution of molecular techniques has become a useful tool for recognition and characterization of fungal species.[27] One of the most frequently used genotyping methods is the random amplified polymorphic DNA (RAPD) analysis for assessing genetic differences in many organisms involving fungi.[28,29] RAPD was very common due to its simplicity and less expensive, speed, and not needed to the previous knowing of DNA sequence, uses only short random primer (<10 bp long). It needs only small amount of genomic DNA and produce large number of markers in a short period.[30] In the present work, this technique was used to determine the genetic relationship among *A. flavus* isolates which collected from different patients.

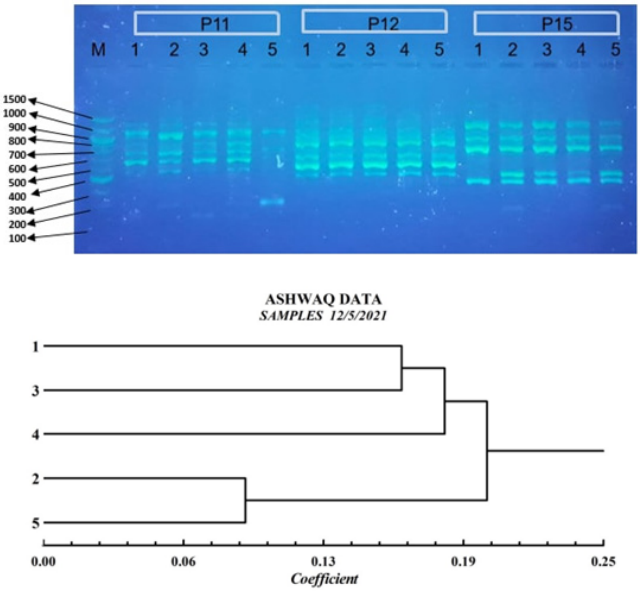

Figure (5): Dendrogram illustrated genetic fingerprint and relationship among *A. flavus* isolates developed from RAPD data.

MATERIALS AND METHODS

Five clinical samples of otomycosis, attending Samarra Hospital and outpatient clinic from the beginning of July 2018 to the end of December 2018 belonging to different ages and genera, formed the subject of this study. Sterile cotton swabs were used for the collection of debris and exudate from external ear. Direct microscopy slides were prepared with 10% potassium hydroxide (KOH) to observe the presence of fungal features (hyphae, septate or aseptate, spores). Swabs were inoculated onto Sabouraud Dextrose Agar (SDA) plates with chloramphenicol, as well as into Sabouraud Dextrose Broth, they incubated at 28ºC and examined for its growth at 24, 48 and 72 hours. Isolates of *A. flavus* were recognized through the appearance of colony, color, size, and morphology, in addition to the microscopic morphology in the lactophenol cotton blue mount or the preparation of slide cultur.[31,32]

DNA Isolation

Fungi mycelium from Sabrouraud Dextrose Broth was used to extract the genomic DNA, and grounded in fine powder using mortar and pestle with liquid nitrogen. Wizard® Genomic DNA Purification Kit (USA) was used for DNA isolation. Nuclei lyses solution was added to the powder for 1-hour and centrifuged at 13000 rpm for 10 second., then washed with phosphate-buffered saline (PBS). Three microliters of RNase was also added to the suspension and incubated at 37ºC for 30 minutes. After cooling the mixture, 200 µL of protein precipitation was added and vortex, then rapidly cooled with ice and centrifuged at 13000 rpm for 4 minutes. The supernatant was transferred to a new Eppendorf tube including isopropanol, then mixed very well and centrifuged for 1 minute at 13000 rpm, the supernatant was discarded and the pellet of DNA was washed with ethanol 70% then centrifuged for 1min at 13000 rpm again. Pellet of DNA was air dried then dissolved with TE buffer. The DNA quality was detection using gel electrophoresis then staining with ethidium bromide to analyze.[33] The purity of the extracted DNA and concentration were checked by the absorbance of diluted DNA solution at 260 and 280 nm using NanoDrop (Thermo Scientific, Germany). The samples were stored at -20°C until used. Nine to ten mer of primers were used for RAPD-PCR analysis. The primers of RAPD were obtained from Operon (Operon Tech., Inc) (Table 1).

PCR Profile

The reaction of PCR was done using the kit of AccuPower PCR premix of BIONEER Corporation (Korea). The tube of premix includes Taq DNA polymerase 1U, 30 Mm of KCL and 1.5 Mm of $MgCl_2$, 250 µM of each dNTPs, Tris-HCl (pH9) 10 Mm per reaction.

The amplification mixture (20 µL vol.) contained 25 ng of genomic DNA, 10 pmol of each primer and reached to

total volume using sterile water, then tube was vortexed and centrifuged after added the primer and DNA template.

The conditions of PCR cycling were consisted of first denaturation step 1 cycle at 94ºC for 2 minutes and subjected to 40 cycles of the following program (denaturation 92ºC for 1 minutes; annealing 36ºC for 1 minutes; extension 72ºC for 1 minutes and final extension 72ºC for 7 minutes as final extension. The products of amplification were separated by electrophoresis 1.5% agarose gel stained by ethidium bromide (0.01 g/mL) and photographed under UV transilluminator.[34] The size of amplicon was estimated after comparing with a commercial 100 bp DNA ladder on an agarose gel.

RESULTS AND DISCUSSION

Under the present study, five isolates of *A. flavus* were subjected to RAPD –analysis to reveal genetic diversity between different studied *A. flavus* isolates. Initially 30 primers were tested in order to search polymorphism between them. Only 12 primers have been able to access the genetic diversity, and generated a total number of bands (243), included (155) bands were common between the isolates and considered as a main band. The rest of these bands 88 bands were polymorphic bands (Table 2). The average number of bands was (20.25) per primer, and the polymorphic band was recorded (7.33) as average number. The number of fragments for these primers ranged between (10–29) with size differing from (100 to 1500) bp. The difference in the number of amplified bands impacted by mutable factors, like, the sequence of primer and less number of annealing locus in the genome.[35] Some of isolates was distinguished with unique bands (present band or absent band). The occurrence of a unique band for the specific isolate is considered as positive marker, whereas the common band when absent was referred as negative marker. OPA-01 primer produced 23 bands which have molecular size ranged between (260–1500)pb, this primer was distinguished isolate number 3 with a unique band (1000) bp and isolate number 5 with absent band (1300) pb figure (1). The OPA-06 primer and OPB-14 primer were relieved a unique band for isolate number 3 at molecular size (1400, 500)bp, respectively (Figure 1 and 2). While the OPB-20, OPC-16 and OPH-08 primers were distinguished the isolate number 1 with absent bands at molecular size (350, 900, 400)bp respectively figure (2,4), so the isolate number 3 was distinguished with absent band (400)bp for the primer OPE-03 (Figure 3). The primer OPF-05 appeared a unique band (400)bp for isolate 2 (Figure 4). Three absent bands (1500, 600, 500)bp and a unique band (350)bp were appeared for isolate 4 with OPC-08 primer figure (1). The primer OPD-18 was distinguished isolate 5 with a unique band (1500)bp and absent band (1300)bp (Figure 3). Only the OPF-20 primer produced a monomorphic main band across all isolates (Figure 4).

The polymorphism of any primer was studied as the ratio of polymorphic bands to the number of total bands which generated from the same primer.[36] High level of polymorphism was shown with primer OPC-08 (100%), whereas a lesser level of polymorphism (0) was presented for the primer OPF-20. The efficiency of primer ranged between (0–6.17), OPD-03 primer gave the minimum efficiency (4.11) and discriminatory power which calculated for each primer by using two equations[36] were given in Table (2).

The data obtained from RAPD profiles was recorded in the binary matrix as the attendance of band (1) or absence of band (0). To establish the genetic relationships among the isolates, similarity coefficient was used to calculate the genetic distance,[37] and dendogram drawn using UPGMA Algorithm (Unweight Pair Group Method Using Arithmetic Average) was used to draw the phylogenetic tree.[38] The decrease of common band numbers between each isolate was conduct to increasing of genetic distance and vice versa. Table 3 shows the genetic distance range from (0.090 to 0.244). The highest distance (0.224) was scored between isolates number (1 and 5) which indicates that the two isolates are distantly related. Meanwhile the lowest genetic distance (0.090) was obtained between isolate number (2 and 5), which shows that there is high similarity between these two isolates. Convergence among *A. flavus* isolates came from many different reasons like source of isolation, similarity in morphological characteristics, genetic characteristic and other character.

Figure 5 reveals the dendrogram tree among the five isolates of *A. flavus* resulting from the clustering values given in Table (3). The analysis was based on the RAPD banding patterns that were different between any given pair of isolates. Genetic coefficients grouped the five isolates in two main cluster: first main cluster was divided in two sub- cluster: first sub- cluster comprised the isolate number (1) and (3) which are considered as genetically closest, whereas the second sub-cluster comprised isolate number (4), which was far from the rest isolates but closer to the isolate (1) and (3). The second main group includes isolate (2) and (5), this revealed the very strong relationship between them due to the great similarity and least genetic variation.

CONCLUSION

The conclusion of this study, is that there was two distinct main relationships for *A. flavus* by RAPD analysis. RAPD profile appeared to be considerably less variable within the different isolates of *A. flavus*. Among the five isolates 2 and 5 were more closely related than 1, 3 and 4. RAPD-PCR technique can be utilized as a fast, easy, and beneficial method for genetic diversity, however, it must be observed that the choosing of arbitrary primers is an essential point to discovery the maximum level of polymorphism.

REFERENCES

1. Carter GR, Wise DJ, Carter GR. Essentials of veterinary bacteriology and mycology. 6th Edn., Ames, Iowa.
2. Mustafa MA, AL-Samarraie MQ, Ahmed MT. Molecular techniques of viral diagnosis. Science Archives. 2020;1(3):89-92.
3. Hospenthal DR, Rinaldi MG, editors. Diagnosis and Treatment of Fungal Infections. 2015; Available from: http://dx.doi.org/10.1007/978-3-319-13090-3
4. Prasad SC, Kotigadde S, Shekhar M, Thada ND, Prabhu P, D'Souza T, Prasad KC. Primary otomycosis in the Indian subcontinent: predisposing factors, microbiology, and

classification. International journal of microbiology. 2014 Apr;2014.
5. Perfect JR, Casadevall A. Fungal molecular pathogenesis: what can it do and why do we need it?. Molecular principles of fungal pathogenesis. 2006 Apr 17:1-1.
6. Nazeer HA, Khaja SM, Rao SS. Mycology of otomycosis in a tertiary care teaching hospital. Journal of research in medical and dental science. 2015 Jan 1;3(1):27-30.
7. Abdullah SK, Al-Samarraie MQ, Al-Assie AH. Fungi associated with grapevine (Vitis vinifera L) decline in middle of Iraq. Egyptian Academic Journal of Biological Sciences, G. Microbiology. 2015 Dec 1;7(1):53-9.
8. Jackman A, Ward R, April M, Bent J. Topical antibiotic induced otomycosis. International journal of pediatric otorhinolaryngology. 2005 Jun 1;69(6):857-60.
9. Viswanatha B, Sumatha D, Vijayashree MS. Otomycosis in immunocompetent and immunocompromised patients: comparative study and literature review. Ear, Nose & Throat Journal. 2012 Mar;91(3):114-21.
10. Gharaghani M, Seifi Z, Zarei Mahmoudabadi A. Otomycosis in Iran: a review. Mycopathologia. 2015 Jun;179(5):415-24.
11. Jadhav VJ, Pal M, Mishra GS. Etiological significance of Candida albicans in otitis externa. Mycopathologia. 2003 Dec;156(4):313-5.
12. AL-Samarraie[1] MQ, Al-Assie AH. New records of some saprophytic and pathogenic fungi isolated from declining grapevine in Salahaldin Province, middle Iraq. Tikrit Journal of Pure Science. 2014;19(5).
13. Lamoth F. Aspergillus fumigatus-related species in clinical practice. Frontiers in microbiology. 2016 May 17;7:683.
14. Khairallah SH, Byrne KA, Tabbara KF. Fungal keratitis in Saudi Arabia. Documenta ophthalmologica. 1992 Sep;79(3):269-76.
15. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology. 2007 Jun 1;153(6):1677-92.
16. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. Asesinos ocultos: infecciones fúngicas humanas. Ciencia Medicina traslacional. 2012;4:165.
17. Pal M. Veterinary and medical mycology. Directorate of Information and Publications of Agriculture, Indian Council of Agricultural Research; 2007.
18. Hetherington SV, Henwick S, Parham DM, Patrick CC. Monoclonal antibodies against a 97-kilodalton antigen from Aspergillus flavus. Clinical Diagnostic Laboratory Immunology. 1994 Jan;1(1):63-7.
19. Al-Abbasi SH, Al-Majmaei AA, Al-Naqib AT, Hameed AM, AL-Samarraie MQ, H Altaef A. Isolation and identification of some fungi from rhizospheric soils of some wild plants at Samarra University, Iraq. Caspian Journal of Environmental Sciences. 2021 Dec 1;19(5):829-39.
20. Gupta SK, Pereira BM, Singh AB. Survey of airborne culturable and non-culturable fungi at different sites in Delhi metropolis. Asian Pacific journal of allergy and immunology. 1993 Jun 1;11(1):19.
21. Raveneau J. Dent, Borden D. (1990) Cartography: Thematic Map Design. Dubuque, Iowa, W.C. Brown Publishers, Second Edition, 433 p. (ISBN 0-697-07991-0). Cahiers de géographie du Québec [Internet]. 1993;37(101):383. Available from: http://dx.doi.org/10.7202/022352ar
22. St. Leger RJ, Screen SE, Shams-Pirzadeh B. Lack of host specialization in Aspergillus flavus. Applied and Environmental Microbiology. 2000 Jan 1;66(1):320-4.
23. Davari E, Mohsenzadeh M, Mohammadi G, Rezaeian-Doloei R. Characterization of aflatoxigenic Aspergillus flavus and A. parasiticus strain isolates from animal feedstuffs in northeastern Iran. Iranian Journal of Veterinary Research. 2015;16(2):150.
24. Wogan G, Goldblatt L. Aflatoxin–Scientific Background, Control and Implications. Aflatoxin, Academic Press, New York-London. 1969:151-86.
25. Coenen A, Kevei F, Hoekstra RF. Factors affecting the spread of double-stranded RNA viruses in Aspergillus nidulans. Genetics Research. 1997 Feb;69(1):1-0.
26. Gehlot P, Purohit DK, Singh SK. Molecular diagnostics of human pathogenic Aspergillus species.
27. Fredricks DN, Smith C, Meier A. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. Journal of clinical microbiology. 2005 Oct;43(10):5122-8.
28. Chongo G, Gossen BD, Buchwaldt L, Adhikari T, Rimmer SR. Genetic diversity of Ascochyta rabiei in Canada. Plant Disease. 2004 Jan;88(1):4-10.
29. Madhavan S, Paranidharan V, Velazhahan R. RAPD and virulence analyses of Colletotrichum capski isolates from chilli (Capsicum annuum). Journal of Plant Diseases and Protection. 2010 Dec;117(6):253-7.
30. Alanio A, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S. Investigating clinical issues by genotyping of medically important fungi: why and how?. Clinical microbiology reviews. 2017 Jul;30(3):671-707.
31. Riddell RW. Permanent stained mycological preparations obtained by slide culture. mycologia. 1950 Mar 1;42(2):265-70.
32. Pitt JI, Hocking AD. Fungi and Food Spoilage. 1997; Available from: http://dx.doi.org/10.1007/978-1-4615-6391-4
33. Joseph S, David WR. Molecular cloning: a laboratory manual. Gold Spring Harbor, New York. 2001;3:17-3.
34. Weigand F, Baum M, Udupa S. DNA molecular marker techniques. Technical Manual No. 20. ICARDA; 1993.
35. Kernodle SP, Cannon RE, Scandalios JG. Concentration of primer and template qualitatively affects product in RAPD-PCR. Biotechniques. 1993;1:362-4.
36. Graham J, McNicol RJ. An examination of the ability of RAPD markers to determine the relationships within and between Rubus species. Theoretical and Applied Genetics. 1995 Jun;90(7):1128-32.
37. Rohlif, F. J. (1993). Numerical taxonomy and multivariate analysis system. Version 1.80. Zxeter. Software. Setauket. N. Y.
38. Sneath, P. H. A. and Sokal, R. R. (1973). Numerical taxonomy the principles of and practice of numerical classification, Freemon, San Francisco. P.573.