

Molecular characterization of *Aspergillus niger* isolates inciting black mould rot of onion through RAPD

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Abstract

Genetic diversity in black mould rot pathogen (*Aspergillus niger* Van Tieghem) was analyzed using ten isolates collected from different regions of India. The genomic DNA extracted from each isolates of *Aspergillus niger* was subjected to polymerase chain reaction using 40 random decamer primers from OPA and OPC series. Only six primers showed amplifications and selected for the analysis. Out of these, all six primers showed 100 per cent polymorphism. The total number of amplified fragments was 159, with a range of 10 to 38 fragments per primer. Dendrogram generated by pooled molecular data of six RAPD primers formed two clusters namely 'A' and 'B'. The cluster 'A' was divided into AN-1 and AN-4 isolates, Cluster B was divided into two sub-clusters B₁ and B₂. Sub-cluster B₁ included B₁₁ and B₁₂ cluster. Cluster B₁₁ included isolates viz., AN-02, AN-03, AN-05 and AN-06. Cluster B₁₂ included isolate AN-09. Sub-cluster B₂ included B₂₁ and B₂₂ cluster. B₂₁ cluster included isolate AN-07 and B₂₂ cluster included isolates AN-08 and AN-10. The similarity coefficient ranged from 0.037 to 0.56 with all the six primers. Highest similarity (0.56) was observed between AN-5 and AN-6 isolates, while lowest similarity (0.037) was observed between AN-1 and AN-2 isolates. Thus, the molecular characterization of ten isolates of *A. niger* by RAPD revealed existence of variations.

Highlights

- The similarity coefficient ranged from 0.037 to 0.56 with all the six primers. Highest similarity (0.56) was observed between AN-5 and AN-6 isolates, while lowest similarity (0.037) was observed between AN-1 and AN-2 isolates. Thus, the molecular characterization of ten isolates of *A. niger* by RAPD revealed existence of variations.

Keywords: Random amplification of polymorphic DNA, molecular diversity, *aspergillus niger*, black mould rot of onion

Onion (*Allium cepa* L.) is an important vegetable crop widely cultivated and used in the world. After harvest of the bulbs when they are preserved for longer duration, many fungal and bacterial pathogens cause deterioration of bulbs. Among all *Aspergillus niger* inciting black mould rot in onion found predominant. The present study was carried out to ascertain molecular diversity in different isolates of *Aspergillus niger* causing black mould rot of onion collected from various regions of the country.

Materials and Methods

Ten onion bulb samples infected with black mould

rot pathogen (*Aspergillus niger*) were collected from different parts of India {Secundrabad (AN-1), Himmatnagar (AN-2), Jaipur (AN-3), Sangali (AN-4), Ahmedabad (AN-5), Anand (AN-6), Amreli (AN-7), Dahod (AN-8), New Delhi (AN-9) and Valsad (AN-10)}, and the isolations were made to obtain the pure culture of the pathogen by following standard tissue isolation method. DNA extraction was done by CTAB method with slight modification (Niu *et al.* 2008) and DNA was amplified with RAPD - PCR (Randomly Amplified Polymorphic DNA -Polymorphic Chain Reaction) technique using random primers. For the molecular characterization of *A. niger* isolates, the 40 different 10-mer RAPD

primers of OPA and OPC series (Eurofins Genomics India Pvt. Ltd.) were screened for the amplification of template DNA of *A. niger*. The primers that gave clear and polymorphic amplification patterns were used for further analysis of all the 10 isolates of *A. niger*. The amplified DNA fragments for each accession were scored as present (1) or absent (0). Data generated by RAPD primers were used to compile a binary matrix for cluster analysis. Genetics similarity among accessions was calculated according to Jaccard's coefficient.

Table 1: Primers used for RAPD analysis of *A. niger*

Primer	Sequence (5'-3')	Temp. value (°C)	GC Content (%)
OPA5	AGGGGTCTTG	30	60
OPA8	GTGACGTAGG	38	60
OPA12	TCGGCGATAG	36	60
OPC2	GTGAGGCGTC	36	70
OPC7	GTCCCGACGA	36	70
OPC18	TGAGTGGGTG	36	60

PCR Protocol

Preparation of Reaction Mixture

The reaction mixture for RAPD analysis was prepared as under:

Millipore sterilized water	- 18.2 µl
Taq Buffer (10X)	- 2.5 µl
dNTPs (2.5mM each) mix	- 0.5 µl
Taq polymerase (3U/µl)	- 0.3 µl
Primer (10 pmoles/µl)	- 1.0 µl
Template DNA 40 ng/µl)	- 2.5 µl
Total	- 25 µl

The master mix was prepared by adding first millipore sterilized water followed by Taq buffer A 2.5 µl, 0.5 µl dNTPs, 0.3 µl Taq polymerase, 1 µl primer and finally 2.5 µl template DNA was added. The reagents were mixed thoroughly by slight vortex followed by spinning in micro centrifuge. The tubes were then placed in thermal cycler for cyclic amplification. The conditions for amplification were followed as mentioned here under:

Programming for PCR

- Step-1: 94 °C for 4 min. (Initial denaturation)
- Step-2: 94 °C for 1 min. (Denaturation after every cycle)
- Step-3: 36 °C for 1 min. (primer specific annealing temperature)
- Step-4: 72 °C for 1 min. (Extension of annealed primer)
- Step-5: 72 °C for 7 min. (Final Extension) Step 2, 3 and 4 comprised of one cycle and the total reaction was carried out for 40 cycles.

PCR products were run on electrophoresis with known molecular marker (mol wt. 1 kbp or 100+500 bp) in 1.8% Agarose gel.

Data scoring

Data were scored on the basis of presence (1) or absence (0) for analysis. If a product was present in a genotype, it was considered as '1' and if absent considered as '0'. The data were maintained in the excel sheet format for further analysis.

The polymorphism percentage was calculated as per the following method.

Polymorphism (%) = (Total number of bands - Number of monomorphic bands)/Total number of bands × 100.

The data generated by RAPD were analyzed with the software POPGENE 32 version 1.31 (Yeh and Boyle, 1997). Diploid data analysis for dominant marker was performed with the assumption of Hardy-Weinberg equilibrium. Multiple populations were used for the estimation of polymorphic loci, Nei's unbiased measures of genetic identity and genetic distance as well as for Dendrogram construction. A Dendrogram was drawn, based on Nei's (1978) genetic distances using UPGMA. This program is an adoption of program NEIGHBOR of PHYLIP version 3.5c by Joe Felsenstein. The drawing was executed for Multiple Populations.

Results and Discussion

RAPD markers proved to be very informative and useful in monitoring the genetic diversity in the biological entity. Therefore, this technique was employed to detect variability present among ten isolates *i.e.* AN-1, AN-2, AN-3, AN-4, AN-5, AN-6, AN-7, AN-8, AN-9 and AN-10 of *A. niger*.

The present study showed a high level of genetic variability between the isolates of *A. niger*. Total 40 different 10-mer RAPD primers of OPA and OPC series (Eurofins Genomics India Pvt. Ltd.) were screened for the amplification of template DNA of *A. niger*. Among these, 34 primers did not produced amplification at all, while only six primers were able to produce clear banding patterns, and were subsequently used to analyze the entire set of 10 isolates of *A. niger*. The fragment size was detected by comparing the amplicons with a 100 bp Ladder. All the primers showed 100 per cent polymorphism. Analysis of the DNA of ten isolates with six primers showed amplification of total 159 bands, with a range of 10 to 38 bands per primer. Overall, *A. niger* isolates exhibited a moderate level of genetic diversity. The maximum 38 bands were generated by primer OPC-2; whereas, primer, OPA-5, OPA-8, OPA-12, OPC-7 and OPC-18 generated 20, 34, 35, 10 and 22 bands, respectively. The lowest numbers

of bands (10 bands) were generated by primer OPC-7. These results showed the ability of RAPD to discriminate among isolates and suggested their application for species identification. The fragment sizes were detected by comparing the amplicons with a 100 bp DNA Ladder. The purpose of this study was to identify the specific primers which are likely to be efficient in revealing the diversity among the isolates of *A. niger*. The results obtained using six primers have been presented in Table 2.

A common dendrogram generated using the NTSYS software has been collectively discussed for all the six primers.

Pooled RAPD

Dendrogram (Figure 1) based on “Nei’s (1978) unbiased measures of genetic distance (Table 3) by UPGMA method” formed two clusters namely A and B.

Table 2: Details of amplification obtained with different RAPD primers

Sr. No.	Name of primer	Sequence (5'-3')	Annealing temperature (°C)	No. of loci	No. of polymorphic loci	Polymorphism percent (%)	Total No. of bands	PIC* value
1.	OPA5	AGGGGTCTTG	30	07	07	100	20	0.735
2.	OPA8	GTGACGTAGG	38	11	11	100	34	0.875
3.	OPA12	TCGGCGATAG	36	12	12	100	35	0.896
4.	OPC2	GTGAGGCGTC	36	10	10	100	38	0.878
5.	OPC7	GTCCCGACGA	36	06	06	100	10	0.800
6.	OPC18	TGAGTGGGTG	36	09	09	100	22	0.859
Total				55	55	–	159	5.043
Average				9.16	9.16	100	26.5	0.840

*PIC-Polymorphism Information Content

Table 3: Jaccard’s similarity coefficient between ten isolates of *A. niger* based on RAPD

	AN-1 (HYD)	AN-2 (HMT)	AN-3 (RAJ)	AN-4 (MAH)	AN-5 (AHD)	AN-6 (AND)	AN-7 (AMR)	AN-8 (DHD)	AN-9 (DLH)	AN-10 (VLS)
AN-1 (HYD)	1.00									
AN-2 (HMT)	0.03	1.00								
AN-3 (RAJ)	0.13	0.39	1.00							
AN-4 (MAH)	0.25	0.10	0.17	1.00						
AN-5 (AHD)	0.10	0.31	0.40	0.09	1.00					
AN-6 (AND)	0.05	0.33	0.32	0.13	0.56	1.00				
AN-7 (AMR)	0.20	0.10	0.20	0.16	0.19	0.14	1.00			
AN-8 (DHD)	0.09	0.10	0.17	0.16	0.20	0.14	0.30	1.00		
AN-9 (DLH)	0.14	0.25	0.30	0.27	0.32	0.29	0.08	0.19	1.00	
AN-10 (VLS)	0.15	0.23	0.21	0.12	0.14	0.14	0.30	0.33	0.31	1.00

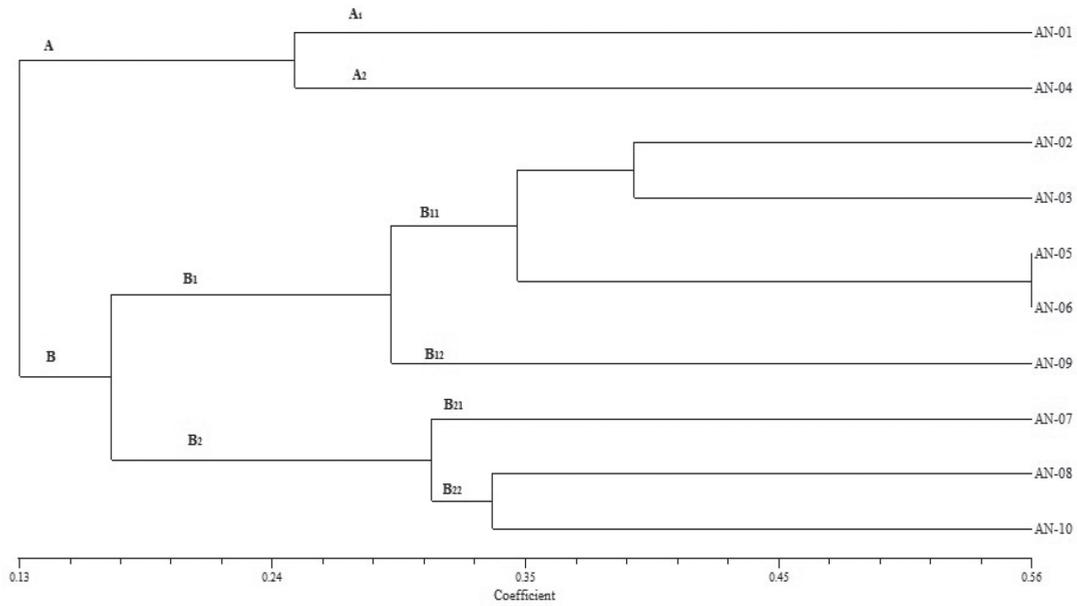
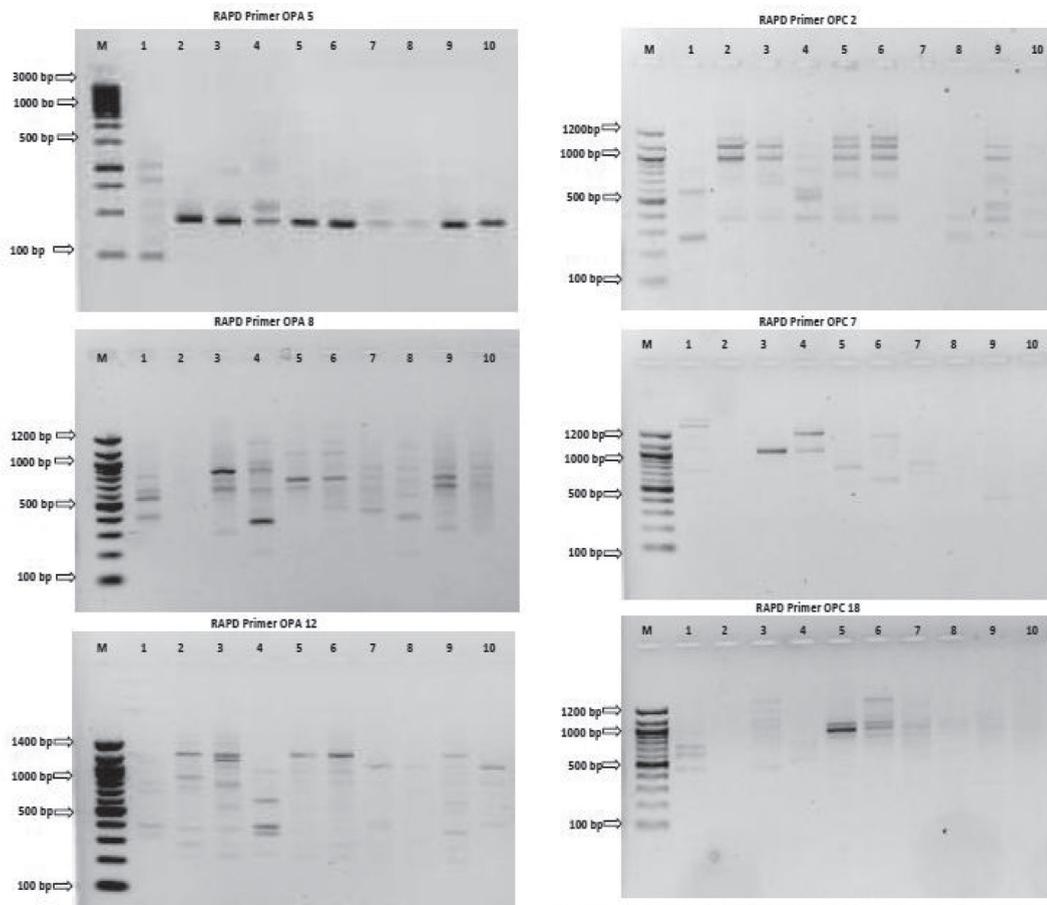


Fig. 1: Dendrogram for ten isolates of *A. niger* based on Nei's (1978) similarity coefficient using UPGMA as the clustering method for RAPD



M-100+bp; 1-AN-1 (HMT), 2-AN-2 (DLH), 3-AN-3 (AMR), 4- AN-4 (DED) 5- AN-5 (AMD), 6-AN-6 (VLS), 7-AN-7 (AND), 8-AN-8 (HYD), 9-AN-9 (RAJ) and 10-AN-10 (MAH)

Plate 1 (a): RAPD amplification patterns of ten isolates of *A. niger* using primers OPA-5, OPA-8 and OPA-12

M-100+bp; 1-AN-1 (HMT), 2-AN-2 (DLH), 3-AN-3 (AMR), 4- AN-4 (DED) 5- AN-5 (AMD), 6- AN-6 (VLS), 7-AN-7 (AND), 8-AN-8 (HYD), 9-AN-9 (RAJ) and 10-AN-10 (MAH)

Plate 1 (b): RAPD amplification patterns of ten isolates of *A. niger* using primers OPC-2, OPC-7 and OPC-18

Cluster A was divided into two sub-clusters A₁ and A₂. Cluster A consisted isolates AN-1 and AN-4 collected from Hyderabad and Maharashtra, respectively.

Cluster B was divided into two sub-clusters B₁ and B₂. Sub-cluster B₁ included B₁₁ and B₁₂ cluster. Cluster B₁₁ included AN-02, AN-03, AN-05 and AN-06 isolates which were collected from Himmatnagar, Rajasthan, Ahmedabad and Anand, respectively. Cluster B₁₂ included isolate AN-09 which was collected from New Delhi. Sub-cluster B₂ included B₂₁ and B₂₂ clusters. B₂₁ cluster included isolate AN-07 collected from Amreli district and B₂₂ cluster included isolate AN-08 and AN-10, which were collected from Dahod and Valsad districts.

The similarity coefficient ranged from 0.037 to 0.56 with all the six primers. Highest similarity (0.56) was observed between AN-5 and AN-6 isolates, which were collected from Ahmedabad and Anand respectively, while lowest similarity (0.037) was observed between AN-1 and AN-2 isolates which were collected from Hyderabad and Himmatnagar, respectively.

Results of present study are in agreement with the results obtained by Ishfaq *et al.* 2014. The genomic DNA of ten isolates of *A. niger* purified and were subjected to RAPD analysis by using 20 different primers. Out of 20 random oligonucleotide primers, primer P2 (5'-ACGGCGTATG-3') was optimized to screen all ten isolates of *A. niger* and showed amplifications of different sizes. Based on amplification patterns, the ten fungal isolates were divided into five groups in dendrogram analysis. Group I contained isolates 658 and 880 (100%), group II contained 0074, 840, 1005 and 1109 (68.38%) isolates, group III has 506 (55.28%) and IV contained isolates 0002 and 744 (36.76%) and group V included 764 (22.54%) based on genomic similarity percentages.

Seven different species of *Aspergillus* with their accession numbers, *A. japonicus* (503), *A. niger* (706), *A. parasiticus* (174), *A. nidulans* (722), *A. flavus* (647), *A. oryzae* (01) and *A. fumigatus* (651) were grown at 24-25°C. Total DNA of fungal samples were extracted manually by CTAB method. Optimized primer GL Decamer B-09: 5' TGGGGGACTC 3' and Primer GL Decamer B-10: 5' CTGCTGGGAC

3' were used for PCR of isolated DNA. *Aspergillus oryzae* and *A. flavus* were 83 per cent similar to each other. *Aspergillus parasiticus* and *A. fumigatus* had 80 per cent similarity, *A. niger* was 82 per cent similar to *A. parasiticus* and *A. fumigatus*. These two groups were 75 per cent similar to each other. *Aspergillus nidulans* showed 60 per cent similarity to *A. oryzae*, *A. flavus*, *A. parasiticus*, *A. fumigatus* and *A. niger*. (Saba and Rabiya, 2012).

Based on RAPD data all 51 isolates of *A. niger* isolated from dried fruit samples (black sultanas, dates, dried figs and prunes) collected from different countries were grouped into four major groups. Greater fungal diversity was found in black sultanas. The 18 isolates from this substrate spanned in all four groups. The isolates collected from prunes, dates, and dried figs spanned into two, three and one group, respectively. Group III accommodated the majority of the isolates. Only group III comprised toxigenic and non-toxigenic isolates. In other three groups, non-ochratoxigenics were found. Good consensus was found between RAPD and b-tub2 sequences data (Ferracin *et al.* 2009).

The primers identified here might be useful to distinguish the isolates of *A. niger* and their characterization and such molecular profiles may prove to be ideal for identification of the virulent strains when a large-scale study is undertaken.

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