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MOLECULAR CHARACTERIZATION OF *PHOMOPSIS VEXANS* ISOLATES OF EGGPLANT OF BANGLADESH

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ABSTRACT

Forty-four isolates of *Phomopsis vexans* were collected from eggplant producing areas of Bangladesh during the 2008-2009 growing seasons. The polymerase chain reaction (PCR) - variable number of tandem repeats (VNTR) technique was used to develop molecular markers. The MR-20 primer amplified template DNA for all isolates studied and resulted in distinct bands. The banding patterns of *P. vexans* isolates were separated into five groups after results with Taq polymerase. The highest genomic DNA concentration 50 ng/µl was found in the isolates of 8, 14, 26, 27 and 38, and the lowest concentration of genomic DNA was found 12.5 ng/µl in the isolates of 1, 2, 5, 15, 17, 22, 30, 31, 33, 34, 36, 40, 41, 42 and 43. Based on the fingerprinting the 44 isolates of *Phomopsis vexans* were categorized into five different groups.

Key words: Molecular characterization, *Phomopsis vexans*, eggplant.

INTRODUCTION

Solanum melongena L. (2n = 24) is also known as eggplant, aubergine, brinjal and Guinea squash. It is the only economic host of *Phomopsis vexans* and the disease is variously known as tip over, stem blight, canker, leaf blight or spot and fruit rot; damping off also can take place. Leaf spots (up to 3 cm diam.) are conspicuous, irregular in outline and may coalesce; lower leaves may be affected first. In stem lesions, the cortex dries and cracks, plants become stunted and girdling cankers cause death of the plant. Fruit spots are pale sunken, conspicuous and may affect the whole fruit; fruit may drop or remain attached, becoming mummified after a soft decay. Pycnidia are abundant. *Phomopsis vexans* has both α and β conidia, pycnidia with short or no pycnidial beaks. It is the sole causal agent of Phomopsis fruit rot of eggplant. Eggplant suffers from 12 diseases of which fruit rot caused by *Phomopsis vexans* (Sacc. and Syd.) Harter is a devastating one. This pathogen remains viable for about 14 months in soil debris and in the seed from infected fruits (Kalda *et al* 1977). This organism is both externally and internally seed borne **(**Singh, 1992). The disease was first reported from Gujrat in 1914 and since then from many parts of India. Occurrence of the disease in Bangladesh has been reported by Fakir (1983) and Ahmad (1987). Crop losses range from 15-20 % in general but 30-50 % in severe case (Das, 1998).

There is no data in the world on the molecular characterization of *Phomopsis vexans* causing fruit rot. The development of molecular techniques was allowed the cataloguing of many genes involved in defense.

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In recent years, both mini-satellite and micro-satellite probes have been applied for DNA fingerprinting of numerous animal, plant and fungal species (reviewed by Rosewich and McDonald 1994, Weising *et al*. 1995). High levels of polymorphism between related genotypes were often observed and the technique found its way to diverse areas of genome analysis, including paternity testing, genotype identification and population genetics. Realizing the importance of this technology, a research program has been conducted on molecular variability of *Phomopsis vexans* isolates.

MATERIALS AND METHODS

Phomopsis vexans infected fruits, leaves and stems were collected from 23 eggplant producing areas in Bangladesh during the growing seasons 2008-2009. The pathogen was isolated from diseased plant parts by plating on acidified potato dextrose agar (APDA). Colonies were sub-cultured onto APDA examined for purity and kept at room temperature (25 $\pm 2^0$C).DNA finger printing of isolates of *Phomopsis vexans* were studied.

Cultures of 44 isolates were grown on APDA for molecular characterization of *Phomopsis vexans* isolates of Bangladesh. Mycelium tips of 3-5 days old purified cultures of *P.vexans* were transferred into 500 ml conical flask containing 50 ml potato dextrose (PD) broth medium and placed on electric orbital shaker for 2-3 days (continuous shaking) at 120 rpm at 25$\pm 1^0$C following the procedure of Anon. (1994). Two to three days old mycelium of *P. vexans* from PD broth medium was filtered and squeezed to remove water with the help of muslin cloth. The squeezed, blotted and flattened mycelium of *P. vexans* was folded into an Al-foil paper and frozen at - 20 ^{0}C followed by drying in a freeze dryer. DNA was extracted from 44 isolates following the methods of Raeder and Broda (1985) and Anon. (2001). Dried mycelium was ground to powder with the help of a pestle and mortar. About 25 mg powdered mycelium was poured into a 1.5 ml centrifuge tube and suspended in 800 µl of extraction buffer (250 mM NaCl, 100 mM Tris-HCl, 100 mM EDTA, all Sigma) by stirring on a Vortex mixer. Eighty (80) micro-liters of 10 % sodium dodecyl sulphate (SDS) was added to the centrifuge tube containing the powdered mycelium and extraction buffer, mixed by inverting a few times and incubated for 30 minutes at 65^{0}C in a water bath. After incubation, 352 µl of 3 M sodium acetate (pH 5.2) was added into the centrifuge tube containing mycelium, extraction buffer and SDS, mixed gently and kept in ice for 15 minutes to burst the cells and denature the proteins. This mixture was centrifuged in a refrigerated centrifuge (Hawk 15/05) at 13000 XG at 4^{0}C for 10 minutes and the water-soluble supernatant was collected and transferred into a new centrifuge tube. Five hundred micro-liter chloroform was added to the new centrifuge tube and mixed in thoroughly on the vortex mixer. The mixture was centrifuged at 13000 XG at 4 ^{0}C for at least 30 minutes. Chloroform precipitates the protein and long centrifugation separates long chain polysaccharides from the DNA. The upper aqueous phase (the DNA solution) was collected into clean tubes very carefully with the help of a pipette without disturbing the interface and this protein containing interface with the organic phase layer beneath the interface was discarded. Precipitation of DNA was done following the procedure of Anon. (2001). Two volumes of pure ethanol were added into the DNA solution and the mixture was kept on ice for 10 minutes to allow the DNA molecules to aggregate. The DNA was precipitated down by centrifugation at 13000 XG at 4 ^{0}C for 5 minutes

to the bottom of the centrifuge tube as a pellet. Then the supernatant was discarded with the help of a wide bore pipette and the centrifuge tube with the DNA pellet was preserved. Two hundred micro-liter of 70 % ethanol was added into the centrifuge tube and stirred on a Vortex mixer for drying the DNA pellet. The tube was then centrifuged at 13000 XG at 4^0C for two minutes and the ethanol was discarded with the help of a micropipette. The DNA pellet was dried in a vacuum desiccators for two minutes and re-suspended in the required volume (depending on the size of DNA pellet) of Tris-EDTA (TE) buffer (10 mM Tris, pH-8.0, 1 M EDTA, all Sigma). The solution was preserved at -20^0C for further experiments. The DNA was assessed following the procedure of Anon. (2001). Two micro-liters of 6X gel-loading dye were mixed with 8 µl of each of the genomic DNA solution in separate centrifuge tubes for each of the isolates.

For preparation of 180 ml of a 0.8 % agarose gel an amount of 1.44 g agarose was weighed in a conical flask and 180 ml of 1X Tris-Acetate EDTA (TAE) was poured into the conical flask and the mixture was heated in a microwave oven until the agar was dissolved fully. The melted agarose gel was cooled down under the flow of tap water and poured gently in the gelcast immediately after, a comb was inserted into the gel tank for making wells. The gel was allowed at-least one hour for solidification before the comb was gently removed. The gel was submerged into the 1X TAE running buffers in the gel tank.Ten micro-liter of each genomic DNA sample was loaded separately into the wells of the 0.8% agarose gel, as well as 5 µl standard molecular weight marker (λ DNA cut by *Hin*dIII + *Eco*R1) and run by electrophoresis at 120 millivolts for one hour for moving the negatively charged DNA towards the anode. After finishing electrophoresis, the gel was stained with 0.5µg/ml ethidium bromide solution for one hour.

The stained agarose gel was rinsed with water for de-staining, illuminated by placing on an UV-trans-illuminator and photographed for assessing the DNA concentration and quality in comparison with the standard molecular weight marker. After estimating the DNA concentration, 1 ng/µl, 5 ng/µl and 20 ng/µl DNA solutions were prepared by adding the required amount of sterile distilled water (SDW). The diluted DNA solution and the original stock were stored in a refrigerator at -20^0C for other experiments. Two concentrations of each of $MgCl_2$, primer and *Taq* polymerase enzyme was used for a series of experiments and selected concentrations were used for the rest of the study. The DNA was amplified by a polymerase chain reaction (PCR). A master-mix of 20 µl was prepared. Then 19 µl of this master-mix was aliquot into an 0.5 ml PCR tube and 1 µl of genomic DNA (5 ng/µl) was added into it. One drop of mineral oil was also added to each PCR tube. The PCR tubes were fitted into the blocks of a PCR machine (Eppendorf Mastercycler personal). The PCR machine was then run under VNTR-PCR protocol with a lid temperature 103 ^{0}C.

a) 94 ^{0}C for 2 minutes
b) 94 ^{0}C for 20 seconds
c) 45 ^{0}C for 45 seconds
d) 72 ^{0}C for 2 minutes
e) Go to b and repeat b, c and d 35 times
f) 72 ^{0}C for 5 minutes and hold at 4 ^{0}C

The tubes with the PCR products were taken out the PCR machine; 2 µl of 6X loading dye were added and mixed in thoroughly. Amplified PCR products were separated on agarose.

The MR (GAG GGT GGC GGT TCT) primer was used in the PCR.Electrophoresis of the gel and its staining and the photographing of the DNA bands were done.

RESULTS AND DISCUSSION

The lowest concentration of genomic DNA was found 12.5 ng/µl in the isolates of 1, 2, 5, 15, 17, 22, 30, 31, 33, 34, 36, 40, 41, 42 and 43 (Table 1). The lower genomic DNA concentration was found 25 ng/µl in the isolates of 3, 4, 6, 9, 10, 12, 13, 16, 19, 20, 21, 23, 24, 25, 28, 29, 37, 39 and 44, higher genomic DNA concentration was found 37.5 in the isolates of 7, 11, 18 and 32 and the highest 50 ng/µl was found in the isolates of 8, 14, 26, 27 and 38(Table 1). DNA samples of all isolates and one negative control (without DNA template) were used in the Polymerase Chain Reaction (PCR) analysis where all showed the expected banding patterns excluding negative control. The negative control (the reaction mixture without DNA) did not reveal any amplification, whereas the positive control (*P. vexans* DNA) showed strong positive PCR product amplification. The primer MR-20 was used to detect the DNA fingerprint of 44 isolates of *Phomopsis vexans*. The DNA fingerprinting patterns for the isolates of 1, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 32, 34, 36, 37, 38, 39, 42, 43 and 44 were identical and so these showed that they came from closely related strains. One band missing in the sample no.6, 7 and 41 compared to the samples no.1, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 32, 34, 36, 37, 38, 39, 42, 43 and 44. In case of samples 26, 31and 40, 3 band is less visible and one band is more visible than the sample 1, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 32, 34, 36, 37, 38, 39, 42, 43 and 44. A prominent band is missing in the middle portion of the samples 2 and 25 than the samples 1, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 32, 34, 36, 37, 38, 39, 42, 43 and 44. But samples 30, 33, and 35 have an extra prominent band in the lower portion than the samples 1, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 32, 34, 36, 37, 38, 39, 42, 43 and 44 (Fig. 1a and 1b).

Dendogram analysis of isolates based on fingerprinting divided the isolates into five clusters. Isolates 26, 31 and 40 are in group five, isolates 2 and 25 are in group four, isolates 30, 33 and 35 are in group three, isolates 6, 7 and 41 are in group two and rest of 33 isolates are in group one (Fig. 2).

The distribution of 44 isolates of *Phomopsis vexans* were grouped into 5 on the basis of DNA fingerprinting have been shown in different locations (Table 2). Isolates 1, 8, 12, 21 and 35 were collected from different sites of Mymensingh district where isolates 1, 8, 12, 21 were in group one and isolates 35 in group three. Isolates 2, 5, 7 and 26 were collected from 4 locations of Jamalpur district where isolate 5 was in group one, isolate 26 in group-3, isolates 2 and 7 in group 4. From Chittagong district, isolates 3, 9, 22, 28 and 36 were collected and all of 5 isolates were in group one. Isolates 4, 6, 13, 17 and 18 were collected from 5 different rural areas of Sherpur district, among them 4, 13, 17 and 18 were in group one and isolate 6 in group two. Isolates 10 and 15 were collected from two different fields of Comilla and both were in group one. Isolates 11, 16, 19, 20, 29, 32 and 40 were collected from Lalmonirhat district where isolate 40 was in group 5 and rests of isolates in group one. Isolates 14, 31 and

39 were collected from Narayangonj district where 14 and 39 were in group one and 31 in group 5. Isolates 23 and 30 were collected from Rajshahi and 23 was in group one and 30 in group 3. Isolate 24 was collected from Bogra that was in group one. Isolates 25, 27, 33, 42 and 44 were collected from Faridpur where isolates 27, 42 and 44 were in group one and 25 was in group 4 and 33 in group 3. Isolates 34 and 38 were collected from Jessore and both were in group one. Isolate 37 was collected from Dhaka and it was in group one. Isolates 41 and 43 were collected from Rangpur where isolates 43 was in group one and isolate 41 in group two (Table 2).

DNA fingerprinting enabled us to select representative pathogen strains of *P. vexans*. This study is the first to describe this pathogen's molecular characteristics. Forty four isolates were identified, all belonging to one species but demonstrated wide molecular diversity. According to DNA fingerprinting, 44 isolates have been categorized into five groups. Piolio *et al.* (2003) reported that *Diaporthe/Phomopsis* isolates could be grouped in four major taxa: (i) *Diaporthe phaseolorum* var. *meridionalis,* (ii) *Diaporthe phaseolorum* var. *caulivora*, (iii) *Diaporthe phaseolorum* var. *sojae* and (iv) *Phomopsis longicola.* In addition to distinguishing interspecific and intraspecific variability, molecular markers allowed the detection of differences among the isolates within the same variety. So the present findings corroborate with the report of Piolio *et al. (*2003) although they did not work with *P vexans*.

DNA removed from mycelia after chloroform extraction and ethanol precipitation and PCR inhibitors that could not be removed by repeated washing with 70% ethanol or repeated DNA replication. To reduce these contaminants, 100% ethanol was used instead of 70% ethanol to recover DNA. DNA recovered with double hellix and the target DNA sequence was consistently amplified from fungal DNA extracts. However, the fact that it was necessary to dilute this DNA for consistent amplification of the target DNA sequence indicated that some PCR inhibitors were still present. The addition of white milk to the PCR reaction allowed the consistent amplification of the target DNA fragment from undiluted DNA extracts from mycelium. The use of white milk to overcome PCR inhibitors has been previously described, although the mechanism by which this occurs is not known.

The MR-20 primer was not species specific i.e. this primer also directed the amplification of an appropriate rice pathogen *Bipolaris oryzae*, *Erwinia anans* etc. However this should not result in false positives in *P. vexans* mycelia assays because these fungi are not associated with eggplant. Alternatively, a more specific MR-20 primer could be developed. New primer can easily be incorporated into this PCR based assay, thereby providing a means for continually updating and improving the specific and sensitivity of the assay.

Table 1. Molecular behaviour of *Phomopsis vexans* isolates

Sl. No.	Isolates No.	Amount of TE added (µl)	DNA concentration (ng/µl)	Primer	PCR	Grouping

Sl. No.	Isolates No.	Amount of TE added (µl)	DNA concentration (ng/µl)	Primer	PCR	Grouping
1	1	130	12.5	MR-20	Positive	01
2	2	100	12.5	MR-20	Positive	04
3	3	130	25	MR-20	Positive	01
4	4	100	25	MR-20	Positive	01
5	5	200	12.5	MR-20	Positive	01
6	6	150	25	MR-20	Positive	02
7	7	100	37.5	MR-20	Positive	02
8	8	200	50	MR-20	Positive	01
9	9	100	25	MR-20	Positive	01
10	10	100	25	MR-20	Positive	01
11	11	90	37.5	MR-20	Positive	01
12	12	90	25	MR-20	Positive	01
13	13	100	25	MR-20	Positive	01
14	14	200	50	MR-20	Positive	01
15	15	100	12.5	MR-20	Positive	01
16	16	130	25	MR-20	Positive	01
17	17	90	12.5	MR-20	Positive	01
18	18	100	37.5	MR-20	Positive	01
19	19	100	25	MR-20	Positive	01
20	20	130	25	MR-20	Positive	01
21	21	100	25	MR-20	Positive	01
22	22	180	12.5	MR-20	Positive	01
23	23	100	25	MR-20	Positive	01
24	24	100	25	MR-20	Positive	01
25	25	90	25	MR-20	Positive	04
26	26	90	50	MR-20	Positive	05
27	27	200	50	MR-20	Positive	01
28	28	100	25	MR-20	Positive	01
29	29	100	25	MR-20	Positive	01
30	30	100	12.50	MR-20	Positive	03
31	31	100	12.50	MR-20	Positive	05
32	32	130	37.50	MR-20	Positive	01
33	33	120	12.50	MR-20	Positive	03
34	34	200	12.50	MR-20	Positive	01
35	35	200	12.5	MR-20	Positive	03
36	36	90	12.50	MR-20	Positive	01
37	37	100	25	MR-20	Positive	01
38	38	200	50	MR-20	Positive	01
39	39	90	25	MR-20	Positive	01
40	40	100	12.50	MR-20	Positive	05
41	41	100	12.50	MR-20	Positive	02
42	42	90	12.50	MR-20	Positive	01
43	43	200	12.5	MR-20	Positive	01
44	44	130	25	MR-20	Positive	01
45	Negative	-	- control	MR-20	Negative	-

Sl. No.	Isolates No.	Amount of TE added (µl)	DNA concentration (ng/µl)	Primer	PCR	Grouping
	control					

MR-20 (5’ GAGGGTGGCGGTTCT 3’)

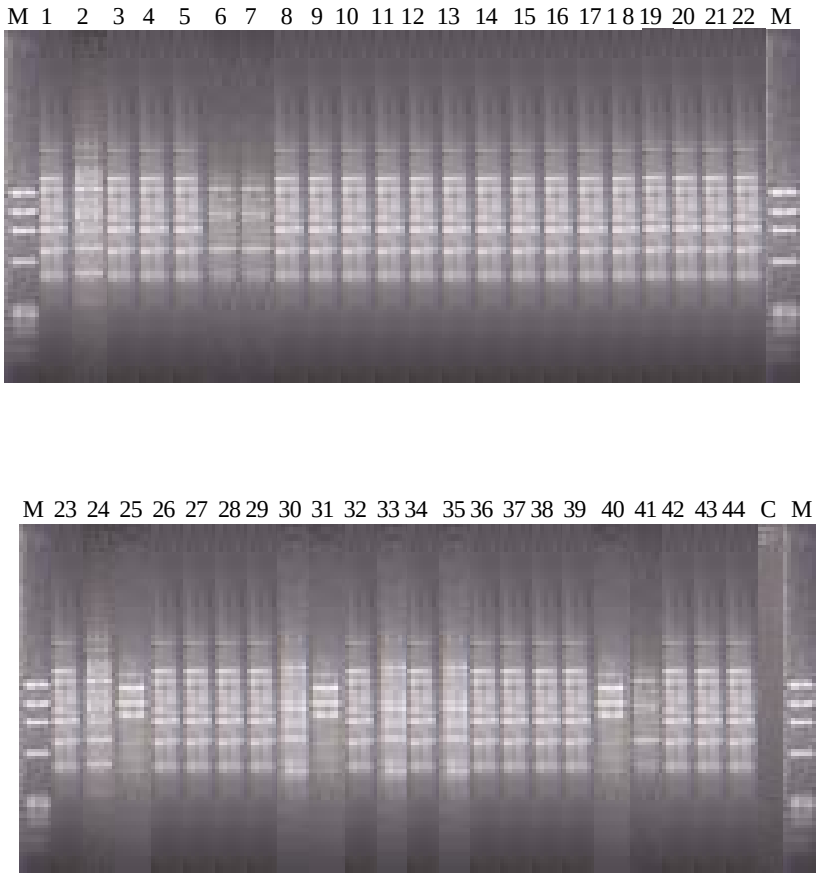
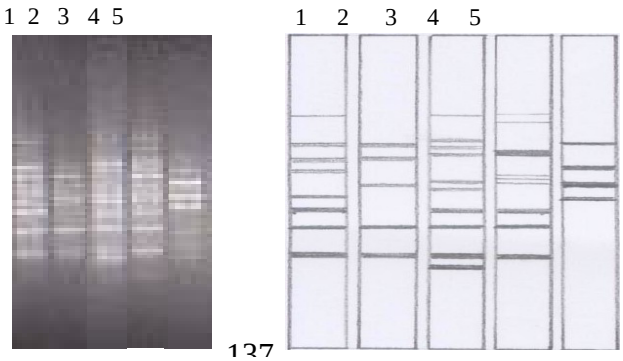


Fig.1a. PCR amplification of variable number of tandem repeats (VNTR) from 44 isolates of *Phomopsis vexans* using primer MR. [Lanes: M. *Phi* x 174 DNA/*Hae* III molecular weight markers. 1-44, isolates as numbered; C, No DNA template (negative control).



Original Typical (Sketch)

Fig. 1b. Five different molecular groups of *Phomopsis vexans* isolates.

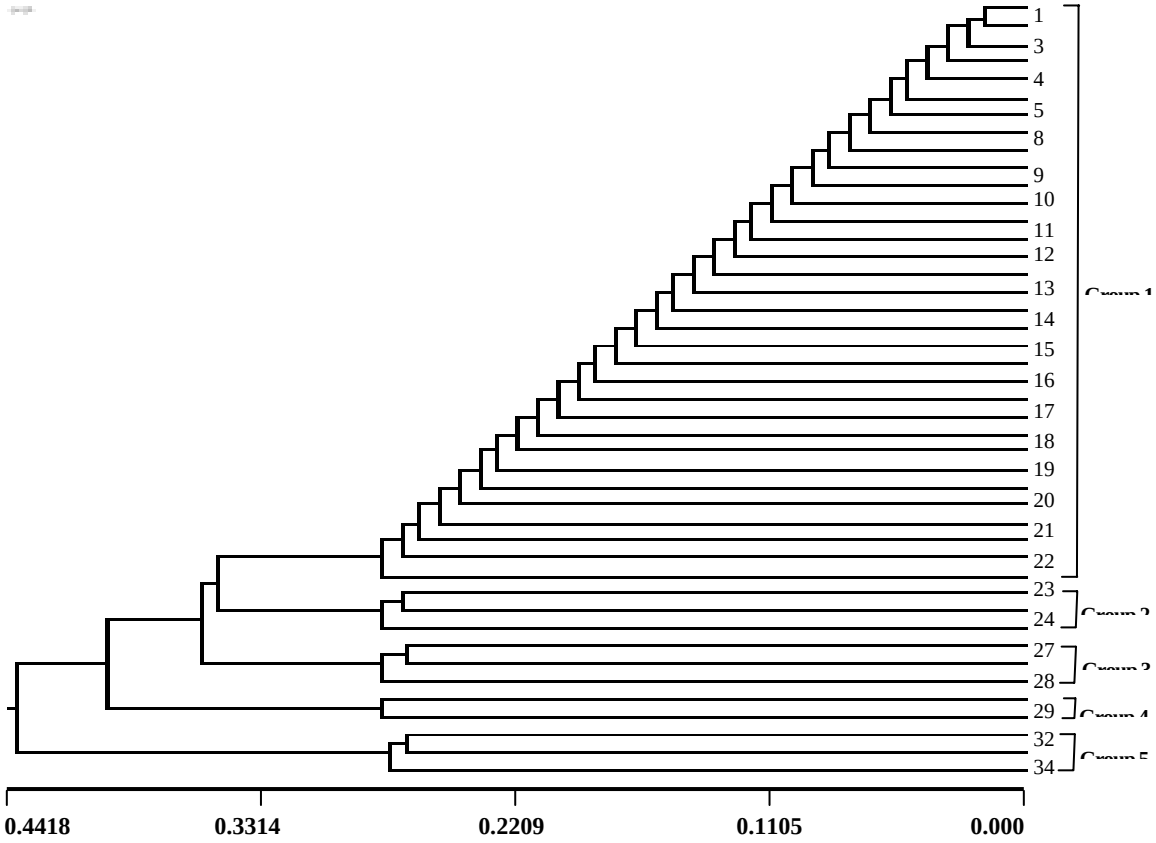


Fig. 2. Dendrogram of *Phomopsis vexans* isolates by VNTR assessed on agarose gel using primer MR-20 based on Nei's (1972) genetic distance.

Table 2. Distribution of molecular groups of *Phomopsis vexans* isolates in different locations of Bangladesh

Locations	Group No					Total
	Group-1	*Group-2*	Group-3	Group-4	Group-5	
Mymensingh	1,8,12,21	-	35	-	-	5
Jamalpur	5	-	-	2,7	26	4
Chittagong	3,9,22,28,36	-	-	-	-	5
Sherpur	4,13,17,18	6	-	-	-	5
Comilla	10,15	-	-	-	-	2
Lalmonirhat	11,16,19,20,29,32	-		-	40	7
Narayangonj	14,39	-	-	-	31	3
Rajshahi	23	-	30	-	-	2
Bogra	24	-	-	-	-	1
Faridpur	27,42,44	-	33	25	-	5
Jessore	34,38	-	-	-	-	2
Dhaka	37	-	-	-	-	1
Rangpur	43	41	-	-	-	2
Total	33	2	3	3	3	44

The PCR based assay developed for the detection of *P. vexans* on infested eggplant fruit is accurate, sensitive and reliable. Because it has the 3-5 days incubation period, this test requires approximately 29 days compared to 10 days for the freezer-blotter test. Another advantage of PCR based assay is that it does not rely on visual identification of *P. vexans* on eggplant fruit, which is complicated by the frequent occurrence of morphologically similar saprobes on eggplant fruit. Thus this PCR based assay has the potential to be used for routine testing of eggplant fruits for *P. vexans*. Furthermore, this type of test may have applicability for the detection of other fungal pathogens on seed or fruit of other crops, once appropriate primer is developed.

The variability in PCR results of the 44 isolates may be due to variations in inoculum loads in the samples. In groups 1,2,3,4 and 5, the DNA extracts from *P.vexans* mycelia produced a distinct banding patterns whereas the negative control samples without any DNA did not produce any band. This indicated that the results were not a false positive by PCR contamination. In picture, many bands are generated as the primer anneals in 10ds of different places. Each uses the same primer and conditions but a DNA sample from different isolates. The patterns of group-1 more or less similar that they are in the same group. In case of group 02, one band is missing compared to group 01. Three (3) bands are less visible in group three and one band is missing compared to group 01. Considering group 4, a band is not visible as like as group 01. Group five has an extra band and one band is missing compared to the group 01.

To distinguish interspecific and intraspecific variability of the *Phomopsis vexans*, molecular markers allowed the detection of differences among isolates with the different eggplant varieties. Moreover, molecular analysis was able to classify as 5 different groups or strains of

P.vexans. Thus VNTR analysis overcame the limitation of classifying isolates based on morphological characteristics only. Fernandez and Halin (1996) using RAPD markers, were able to differentiate among taxa within the *Diaporthe/Phomopsis* complex, as well as among isolates within *D. phaseolorum* var. *caulivora*. Zhang *et al*. (1998) also by using PCR-restriction fragment length polymorphism, detected genomic differences among *D. phaseolorum* var. *caulivora* isolates and *D. phaseolorum* var. *mariodionalis* isolates originated from the same areas of Italy and the United States, respectively. In our study, grouping different isolates of *P. vexans* based on similarity of band patterns corresponded with grouping them based on morphological characters. Because of the congruence of both morphological and VNTR groupings and the correspondence among isolates, it was concluded that grouping assignment of the isolates was appropriate. Even when, in our study, 44 isolates were used, both morphological and molecular analysis allowed the separation of the different strains of the *P. vexans* present in the core eggplant producing areas of Bangladesh.

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