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GENETIC DIVERSITY OF *Pseudomonas syringae* pv. *syringae* CAUSING LEAF BLIGHT OF LITCHI IN BANGLADESH

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ABSTRACT

Leaf blight of caused by *Pseudomonas syringae* pv. *syringae* is a severe disease of litchi saplings, which is considered as a threat for raising healthy sapling of the fruit in Bangladesh. A survey was conducted to find out incidence and severity of the disease in five regions of Bangladesh namely Rajshahi, Dinajpur, Mymensingh, Barishal and Khagrachari. The highest disease incidence was recorded in Dinajpur and Kagrachari regions and the lowest in Mymensingh region. On the other hand, the highest disease severity was found in Dinajpur and Kagrachari regions and the lowest in Mymensingh region. Another experiment was conducted to determine genetic diversity of the causal bacterium following molecular study. Twenty five diseased samples of bacterial leaf blight of litchi were collected from 25 locations in five regions and one isolate of *P. syringae* pv. *syringae* was isolated from each sample. The values of pair-wise comparisons of Nei's (1972) genetic distance among5 different locations of *P. syringae* pv. *syringae* were computed. From the UPGMA dendrogram drawn on the basis of Nei's (1972) genetic distances and comparatively the highest genetic distance (0.9163) was observed in Khajrachari vs Barishal, Mymensingh vs Barishal and Rajshahi vs Mymensingh but Dinajpur was close to the Barishal with the least genetic distance (0.3567). Thus, the results indicate that remarkable genetic diversity exists among the isolates of *P. syringae* pv. *syringae* from different origins Bangladesh.

Keywords: Genetic diversity, Leaf blight of litchi, *Pseudomonas syringae* pv. *syringae,* RAPD marker.

INTRODUCTION

Litchi (*Litchi chinensis Sonn* or *Nephelium litchi Cambess*) is the only member of the genus *Litchi* under the family, Sapindaceae. Litchi is famous for its excellent quality, pleasant flavor, juicy pulp with attractive red color. It is also an excellent source of vitamin C (40-90 mg $100g^{-1}$) but it contains insignificant amount

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of protein (0.8-0.9%) fat (0.3%), pectin (0.43%) and minerals especially calcium, phosphorus and iron (0.7%) (Khadiza, 2012). In Bangladesh, the leading litchi growing districts are Dinajpur, Rajshahi, Rangpur, Jessore, Chittagong and Dhaka. In the country, saplings or seedlings of litchi are attacked by four diseases viz. leaf spot, leaf blight, die-back and red rust (Hossain, 2011). Among 37 recorded diseases of fruit nurseries, bacterial leaf blight caused by *Pseudomonas syringae* pv. *syringae* most important one. This is first time record in Bangladesh by (Hossain et al. (2011). In the country, Leaf blight of litchi ultimately caused death of litchi seedlings. The genetic diversity of *P. syringae* pv. *syringae* has not been studied under Bangladesh condition. In view of such information, the present experiment was undertaken to find out the incidence and severity of leaf blight of litchi in major litchi growing areas of Bangladesh, for molecular characterization of the pathogen and to determine the genetic variation among the isolates of *Pseudomonas syringae* pv. *syringae* collected from different regions of the country based on the RAPD marker.

MATERIALS AND METHODS

Survey of leaf blight Incidence and severity

A survey was carried out to find out the incidence and severity of bacterial leaf blight of litchi nurseries in Rajshahi, Dinajpur, Mymensingh, Rangpur and Khagrachari areas. The surveyed nurseries were selected randomly. The incidence of leaf blight was recorded determined following the formulae of Rai and Mamtha (2005) as shown below:

$$\text{Disease incidence (\%)} = \frac{\text{Number of Infected Plant Units}}{\text{Total Number of Units Assessed}} \times 100$$

Disease severity was computed using the following formula (Johnston 2000):

$$\text{Disease severity (\%)} = \frac{\text{Area of Plant Tissue Affected by disease}}{\text{Total leaf area}} \times 100$$

Isolation, Identification, Multiplication and Purification of the bacterial pathogen

The infected portion of the leaf sample was cut into small pieces, washed with sterile distilled water, and plated on nutrient agar (NA). Plates were incubated at 28°C for 24 hrs. Cream off white colored colony of bacteria appeared on NA medium after incubation period was to plates containing NA. Pure culture of the bacteria was prepared following by streaking and single colony method (Kelman, 1954). The bacterium was characterized by a series of biochemical test viz. KOH solubility test, Gram staining test, Kovac's oxidase test, Temperature sensitivity test,

Levan test, Sugar utilization test, Arginine dihydrolase activity, Catalase test and Pectolytic test and identified as *P. syringae* pv. *syringae*. Then the bacterial isolates were multiplied on using NA medium. The petridishes containing NA media were inoculated with the pure culture of the bacterium with the help of flame sterilized platinum wire loop. All the inoculated plates were incubated at room temperature for 24 hrs. In case of contamination, the bacterial cultures were purified by streaking a single colony of each isolate by sub culturing on NA medium

Molecular characterization of *Pseudomonas syringae* pv. *Syringae*
DNA extraction of bacterial isolates

The bacteria for DNA extraction were cultured in Peptone broth medium at 27°C for 18 hours with shaking on an electrical shaker. Genomic DNA of each isolates were extracted following standard protocol as described by (Ferdous, 2012). Exactly 1.5 ml of bacterial culture was transferred into the eppendorf tube and spin at 5,000 rpm for 5 minutes in a centrifuge machine. All the supernatant were removed and the cell pellet was leaved in the tubes. One milliliter of 2M NaCl solution was added to each tube and the pellet was resuspended by vortexing on a Vortex. The tubes containing the cell suspension were centrifuged at 5,000 rpm for 5 min. The supernatant was removed after centrifugation and the pellet was resuspended in 525 µl of TE buffer and 60 µl of 20% SDS was added in the mixture in each tube and mixed gently. Exactly 15 µl of Proteinase K (200 µg ml^{-1}) was added and incubated for 1hr at 37°C for the digestion of proteinaceous substances. One hundred microliter of 5 M NaCl was added to the mixture of each tube and mixed well. Eighty microliter of CTAB was added and mixed well followed by incubation in the heating water bath at 65°C and mixed gently every 5 minutes. About 750 µl of Chloroform-Isoamyl Alcohol (24:1) was added in each tube and mixed by inverting the tube for several times. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant was transferred into a new tube very carefully to avoid the chloroform in the supernatant. Then equal volume of Phenol-Chloroform (27:25:1) was added in every tube and mixed well by inverting the tube. The mixture was centrifuged at 12,000 rpm at 4°C for 10 minutes. All the supernatant was transferred from the tubes into new tubes. Exactly 500 µl of isopropanol was added into the supernatant and mixed well by inverting the tubes. The tubes were centrifuged at 13,000 rpm at 4°C for 20 minutes. One milliliter of 70-75% ethanol was added and centrifuged at 12,000 rpm at 4°C for 15 minutes. Ethanol was removed with the help of an aspirator and the pellet was allowed to dry in the air for 5 minutes. The pellet was resuspended in 20 µl of TE buffer and incubated at 65°C for 10 minutes or at room temperature for 10 minutes. The isolated genomic DNA was preserved in a refrigerator at -20°C for further use.

PCR amplification and electrophoresis

The amplification conditions were based on Williams et al. (2000) with some modification. PCR reactions were performed on each DNA sample in a total volume

of 10 µl containing Template DNA 2.0 µl, Tag buffer 1.0 µl, dNTPs 1.0 µl, $MgCl_2$-0.6 µl, Ampli Taq DNA polymerase 0.2 µl, ddH2O 3.2 µl, Primer 2.0 µl. The primer code and sequence of the selected primer used in the experiment was 62538035 and TGCAGCACCG with 70% GC content. The ladder used in this experiment was 100kb. PCR was programmed with an initial denaturing at 92°c for 4 min. followed by 40 cycles of denaturation at 94°c for 1 min, annealing at 37°c for 1 min. and extension at 72°c for 3 min. and the final extension at $72°_C$ for 5 min. in a thermocycler (Master Cycler Gradient, Eppendorf, Germany. PCR product (2 µl) was mixed with 1 µl of bromophenol blue and loaded in 1.4% Agarose gel. Electrophoresis was carried out at 120v for 40 min and soaked in ethidium bromide (9ml water +1ml stock) for 15-20 min.

Documentation of the DNA samples and data analysis

After electrophoresis the image of the gel was documented through UV transilluminator system. The dendrogram was constructed using the POPGENE (Version 1.31) computer program 30.

RESULTS AND DISCUSSION

Identification of disease

Results of biochemical or physiological tests performed for identification of the causal bacteria of leaf blight of litchi are summarized in table 1. All tests showed positive reaction and the causal bacterium was idenfied as *P. syringae* pv. *syringae.*

Incidence and severity of litchi leaf blight

During the survey, occurrence of leaf blight was recorded from every litchi sampling nursery in each of the regions included in the present survey. The highest disease incidence was recorded in Dinajpur and Kagrachari regions and the lowest was recorded from Mymensingh region. On the other hand, the highest disease severity was found in Dinajpur and Kagrachari regions and the lowest severity were found in Mymensingh region (Figure 1)

Molecular characterization of *P. syringae* pv. *syringae* collected from five regions

Primer produced total 9 bands all of which (100.00%) were considered polymorphic in *P. syringae* pv. *syringae* collected from litchi nursery of Barishal region (Figure 2A). It produced total 2 bands of which 2 bands (100.00%) were considered as polymorphic in *P. syringae* pv. *syringae* collected from litchi of Dinajpur location (Figure 2B). It produced a total of 9 bands. Of which 8 bands (90.00%) were considered as polymorphic in *P. syringae* pv. *syringae* collected from litchi of Khagrachari location (Figure 2C). It produced a total 9 bands. All of which (100.00%) were considered as polymorphic in *P. syringae* pv. *syringae* collected from litchi of Mymensingh region (Figure 2D). It produced a total of 10

bands. Of which 100% bands were considered as polymorphic in *P. syringae* pv. *syringae* collected from litchi growing regions of Rajshahi (Figure 2E).

Gene diversity and frequency of polymorphic loci of different regions in Bangladesh

Within the isolates from this primer genetic diversity values are given in table 2. Overall observed number of average alleles (*na) is 2 with 5 sample size. Effective number of average alleles (*ne) [Kimura and Crow, 1964], Average Nei's (1973) gene diversity (*h) across all isolates for all loci studies and Average Shannon's Information index (*I) (Lewontin, 1972) across all isolates for all loci are recorded in table 2 & figure 2

Gene flow and co-efficient of gene differentiation of different regions in Bangladesh

Nei's analysis of gene diversity (h) in subdivided isolates estimated the gene flow (Nm), Hardy-Weinberg expectation of average heterozygosity in subpopulation (Ht), obtained heterozygosity (HS) and Co-efficient of gene differentiation (Gst) was recorded in table 3 for all regions of Bangladesh.

Genetic distance and genetic identity with Dendrogram

Genetic distance and genetic identity in all regions separately and al together are shown in table 4. And in the same way dendrogram was drawn for all regions separately and all together are showed in figure 3. In Barishal region, from the UPGMA dendrogram based on Nei's (1972) genetic distances, isolate 4 was closed to the isolate 5 with the least genetic distance (0.1111). In Dinajpur region, from the UPGMA dendrogram based on Nei's (1972) genetic distances, isolate 4 was highest far from the isolate 5 with the highest genetic distance (1.0000). In Khagrachari region, from the UPGMA dendrogram based on Nei's (1972) genetic distances, isolate 4 was close to the isolate 5 with the least genetic distance (0.0000). In Mymensingh region, from the UPGMA dendrogram based on Nei's (1972) genetic distances, isolate 4 was close to the isolate 5 and isolate 2 was close to the isolate 5 with the least genetic distance (0.0000). In Rajshahi region, from the UPGMA dendrogram based on Nei's (1972) genetic distances, isolate 4 was close to the isolate 1 with the least genetic distance (0.2000).

Finally, the values of pair-wise comparisons of Nei's (1972) genetic distance between 5 different location of *Pseudomonas syringae* pv. *syringae* were computed. From the UPGMA dendrogram based on Nei's (1972) genetic distances, Dinajpur was close to the Barishal with the least genetic distance (0.3567).

CONCLUSION

The results indicate that the lower or higher level of genetic distance exists among the isolates of *Pseudomonas syringae* pv. *syringae* causal agent of leaf blight of litchi with their different origin. Leaf blight of litchi caused by *Pseudomonas*

syringae pv. syringae seems to be a great threat for raising quality and healthy litchi saplings in order to get higher fruit production. The findings of the present study would definitely be useful to design a comprehensive molecular based study of biodiversity of the pathogen *Pseudomonas syringae pv. syringae* and to adopt a proper management strategy suitable for the integrated disease management programs.

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Table 1. Biochemical tests of *Pseudomonas syringae* pv. *syringae* isolated from leaf samples of litchi infected with leaf blight

Test	Reaction	Inference
Pathogenicity test	+ ve	*Pseudomonas syringae* pv. *syringae*
KOH test	+ ve	*Pseudomonas syringae* pv. *syringae*
Kovac's test	- ve	*Pseudomonas syringae* pv. *syringae*
Levan test	+ ve	*Pseudomonas syringae* pv. *syringae*
Arginine test	- ve	*Pseudomonas syringae* pv. *syringae*
Catalase test	+ ve	*Pseudomonas syringae* pv. *syringae*
Sugar utilization test	+ ve	*Pseudomonas syringae* pv. *syringae*
Potato soft rotting test	+ ve	*Pseudomonas syringae* pv. *syringae*

Table 2. Nei's (1973) gene diversity with Shannon's Information index from 5 isolates of *Pseudomonas syringae* pv. *syringae* isolated from leaf blight infected samples of litchi grown five regions of Bangladesh

Location	Total Loci	Sample size	na*	ne*		h*		I*	
				max	min	max	min	max	min
Barishal	9	5	2	1.9231	1.4706	0.48	0.32	0.6730	0.5004
Dinajpur	2	5	2	1.9231		0.48		0.6730	
Khagrachari	10	5	2	1.9231	1.0000	0.48	0.00	0.6730	0.0000
Mymensingh	9	5	2	1.9231	1.4706	0.48	0.32	0.6730	0.5004
Rajshahi	10	5	2	1.9231	1.4706	0.48	0.32	0.6730	0.5004

*na = Observed number of alleles,
*ne = Effective number of alleles [Kimura and Crow (1964)]
*h = Nei's (1973) gene diversity,
*I = Shannon's Information index [Lewontin (1972)]

Table 3. Gene flow (Nm) and the proportion of total genetic diversity (Gst) across different RAPD markers with Co-efficient of gene differentiation from 5 isolates of *Pseudomonas syringae* pv. *syringae* collected from litchi of five locations of Bangladesh

Location	Total Loci	Sample size	Ht		Hs	Gst	Nm
			max	min	Average	Average	Average
Barishal	9	5	0.48	0.32	0	1	0
Dinajpur	2	5	0.48		0	1	0
Khagrachari	10	5	0.48	0.00	0	1	0
Mymensingh	9	5	0.48	0.32	0	1	0
Rajshahi	10	5	0.48	0.32	0	1	0

Ht = Hardy-Weinberg average heterozygosity expected in subpopulation
Hs = Hardy-Weinberg average heterozygosity obtained in Sub population
Gst = Co-efficient of gene differentiation
Nm = Estimation of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst;

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) in different isolates pair of *Pseudomonas syringae* pv. *syringae* collected from litchi of five locations of Bangladesh

	Genotypes	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Barishal	Isolate 1	****	0.8889	0.6667	0.3333	0.5556
	Isolate 2	0.1178	****	0.7778	0.2222	0.6667
	Isolate 3	0.4055	0.2513	****	0.2222	0.8889
	Isolate 4	1.0986	1.5041	1.5041	****	0.1111
	Isolate 5	0.5878	0.1178	0.1178	2.1972	****
	Genotypes	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Dinajpur	Isolate 1	****	0.0000	0.5000	0.5000	0.5000
	Isolate 2	0.0000	****	0.5000	0.5000	0.5000
	Isolate 3	0.6931	0.6931	****	1.0000	0.0000
	Isolate 4	0.6931	0.6931	0.0000	****	0.0000
	Isolate 5	0.6931	0.6931	0.0000	0.0000	****

Khagrachari	Genotypes	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
	Isolate 1	****	0.7000	0.2000	0.6000	0.6000
	Isolate 2	0.3567	****	0.3000	0.9000	0.9000
	Isolate 3	1.6094	1.2040	****	0.4000	0.4000
	Isolate 4	0.5108	0.1054	0.9163	****	1.0000
	Isolate 5	0.5108	0.1054	0.9163	0.0000	****
Mymensingh	Genotypes	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
	Isolate 1	****	0.6667	0.3333	0.4444	0.3333
	Isolate 2	0.4055	****	0.4444	0.7778	0.2222
	Isolate 3	1.0986	0.8109	****	0.6667	0.3333
	Isolate 4	0.8109	0.2513	0.4055	****	0.2222
	Isolate 5	1.0986	1.5041	1.0986	1.5041	****
Rajshahi	Genotypes	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
	Isolate 1	****	0.7000	0.4000	0.2000	0.5000
	Isolate 2	0.3567	****	0.7000	0.3000	0.6000
	Isolate 3	0.9163	0.3567	****	0.4000	0.5000
	Isolate 4	1.6094	1.2040	0.9163	****	0.3000
	Isolate 5	0.6931	0.5108	0.6931	1.2040	****
Five Location	Genotypes	Barishal	Dinajpur	Khajrachari	Mymensingh	Rajshahi
	Barishal	****	0.7000	0.4000	0.4000	0.5000
	Dinajpur	0.3567	****	0.5500	0.5500	0.6000
	Khajrachari	0.9163	0.5978	****	0.4500	0.5000
	Mymensingh	0.9163	0.5978	0.7985	****	0.4000
	Rajshahi	0.6931	0.5108	0.6931	0.9163	****

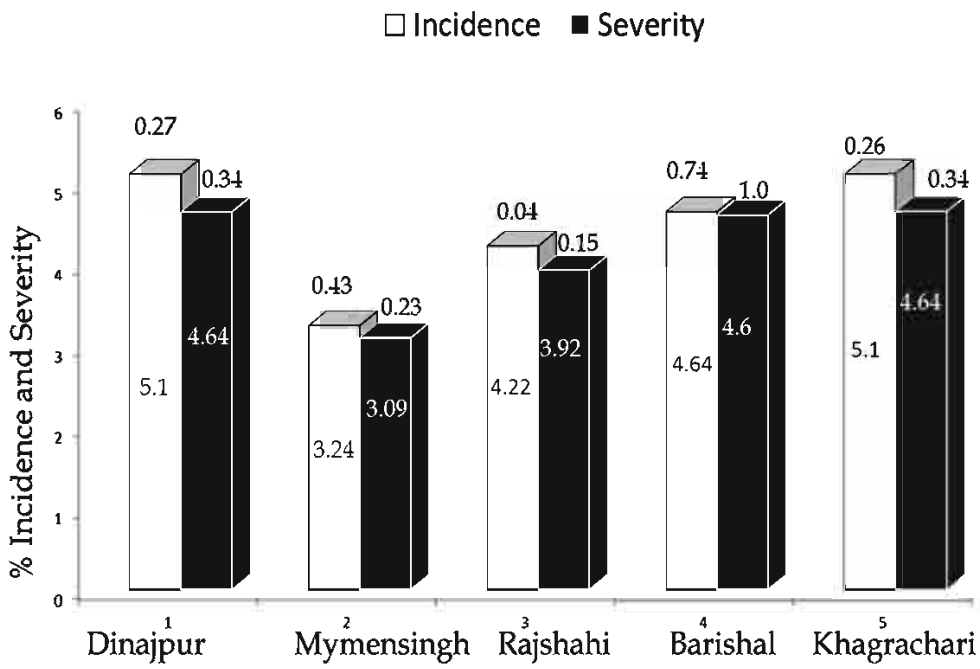

Figure 1. Graphically showing the disease incidence and severity percent of bacterial leaf blight of litchi causing *Pseudomonas syringae* pv. *syringae* (mean value in the legend and SD value on the legend) in different regions of Bangladesh

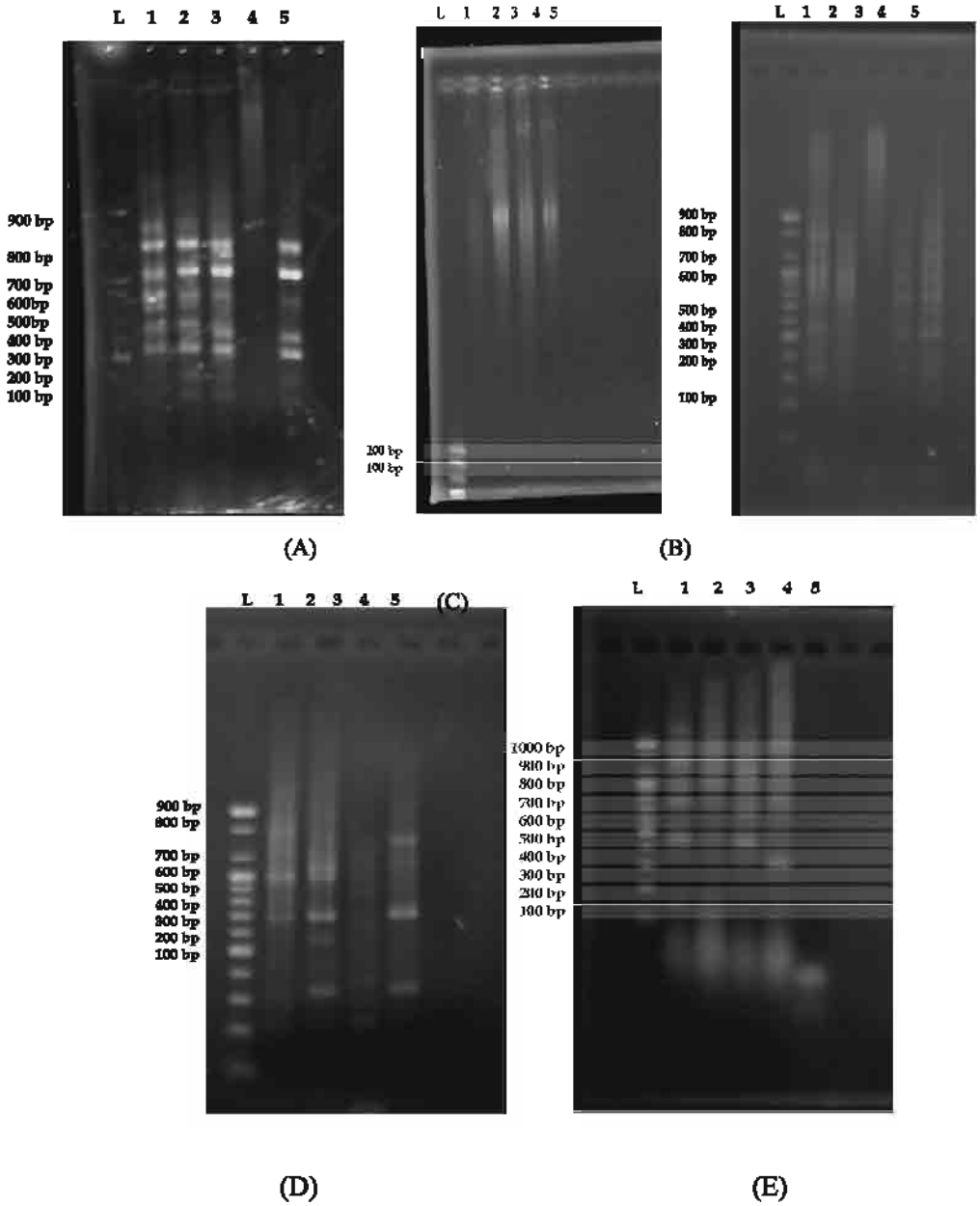

Figure 2. RAPD profile of 5 isolates of *Pseudomonas syringae pv. syringae* using primer 62538038. Molecular weight marker 70.90=85.7nm, 100bp DNA ladder in left side. Lane 1-5: isolates of *P. syringae pv. syringae* collected from litchi of (A) Barishal, (B) Dinajpur, (C) Khagrachari, (D) Mymensingh, (E) Rajshahi location

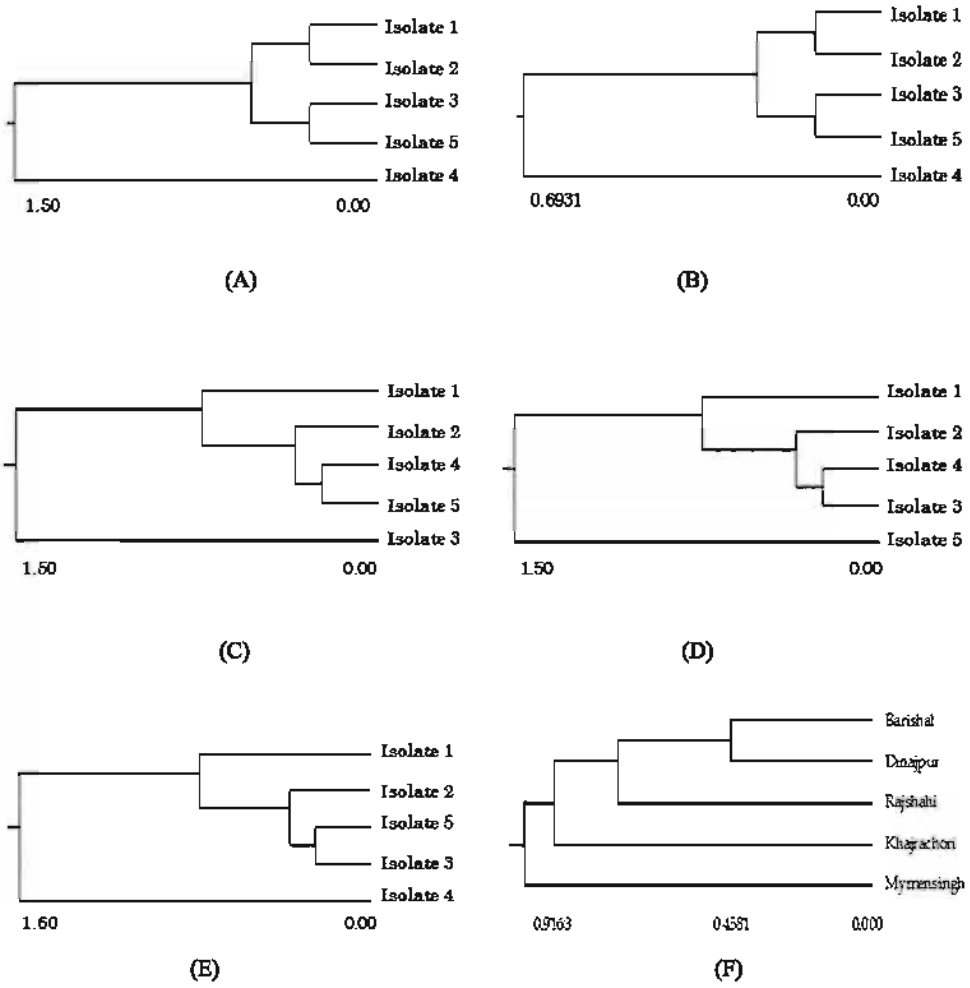

Figure 3.Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Neis's (1972) genetic distance, summarizing data on differentiation of 5 Isolates Litchi blight causing organism *Pseudomonas syringae* pv. *syringae* of Five location according to RAPD analysis in Bangladesh; (A) Barishal, (B) Dinajpur, (C) Khagrachari, (D) Mymensingh, (E) Rajshahi, (F) Five Location