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GENETIC VARIABILITY AMONG THE STRAINS OF *Xanthomonas axonopodis* PV. *CITRI* THROUGH REP-PCR

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ABSTRACT

Citrus canker is one of the most devastating citrus diseases in the world. The Phytopathogen responsible for this disease is *Xanthomonas axonopodis* pv. *citri* (*Xac*). Thirty isolates of *Xac* were isolated from three districts of Uttarakhand state and out of which 13 isolates were selected for genetic diversity study. Pathogenicity test was conducted to identify the pathogen and various morphological and biochemical tests were conducted for characterization of bacterial isolates. PCR based asseys i.e. Repetitive DNA polymerase chain reaction (rep-PCR) fingerprinting using repetitive extragenic palindromic (rep) and enterobacterial repetitive intergenic consensus (ERIC) and BOX primers were used to study genetic diversity. All the 13 isolates differentiated into 13 distinct banding patterns (haplotypes) by rep-PCR technique. The generated banding patterns by the rep-PCR based assays were compared among all the *Xac* isolates. UPGMA cluster analysis of the fingerprints generated by BOX, REP AND ERIC primers revealed considerable diversity among the isolates collected from different areas of Uttarakhand. Cluster analysis grouped the isolates into two main clusters (A & B) with 28 per cent similarity. The results on molecular characterization revealed that the isolates collected from different locations of Uttarakhand were found diversified, due to different geographic location of isolates.

Keywords: BOX, ERIC, PCR, REP, Repetitive DNA polymerase chain reaction (rep-PCR), *Xanthomonas axonopodis* pv. *citri* (*Xac*)

Citrus fruits are very important in terms of international trade, they are valued both as fresh fruits and processed fruits. The present day citrus is delectable, juicy, seedless and is of great nutritional significance as well (Khan *et al*., 1992). The canker disease is the major biotic constraint for its cultivation. It is caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*). The disease is of great economic importance all over the citrus growing area of the world including India, which adversely affects plant health and fruit development. It has played a significant role in causing extensive losses in nurseries and orchards (Gottwald and Irey, 2007). The disease is believed to have originated in South East Asia, is extremely persistent when it becomes established in an area. It was first reported in India from the state of Punjab (Luthraand Sattar, 1942). Since then its occurrence was recorded in all the citrus growing states of the country (Das, 2003). It become endemic in India and occurs in all the citrus growing areas. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification. Generally DNA-based methods are emerging as the more reliable, simple and inexpensive ways to identify and classify microbes. A simple and efficient DNA fingerprinting method based on Polymerase Chain reaction (PCR) using primers has been developed. Fingerprinting methods used to distinguish between strains within target species are easy to perform and data interpretation is relatively straight forward. Frank *et al.* (1994) used DNA primers corresponding to conserved motifs in bacterial repetitive (REP, ERIC and BOX) elements to show that REP, ERIC and BOX-like DNA sequences are widely distributed in phytopathogenic *Xanthomonas* and *Pseudomonas* strains. REP, ERIC and BOX-PCR (collectively known as rep-PCR) were used to generate genomic fingerprints of a variety of *Xanthomonas* and *Pseudomonas* isolates and to identify pathovars and strains that were previously not distinguishable by other classification methods. The present investigation is made to study genetic diversity within 13 differents strains of *Xanthomonas axonopodis* pv. *citri* isolated from 13 different locations of three districts of Uttarakhand, through REP-PCR genomic fingerprinting with REP, ERIC and BOX primers. All the isolates were identified and further characterized on the basis of pathogenecity, morphological and biochemical tests.

MATERIALS AND METHODS

The present study was carried out in the laboratory. The laboratory experiments were conducted at the Department of Plant Pathology, College of Agriculture,

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G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. The objective was to study the variability in *Xanthomonas axonopodis* pv. *citri*.

Isolation and purification of bacteria

The isolation of bacterium was done as per the method described by Janes (2005). The diseased samples (leaves and fruits) showing typical symptoms of bacterial canker were collected from different locations of Uttarakhand and washed throughouly with tap water. The samples were then surface sterilized with 70% ethanol. After surface sterilization the bacteria were streaked on plates containing modified Wakimoto medium with inoculating loop. The inoculated plates were incubated at 28$\pm$2^{0}C in the BOD incubator for 72 hours. The pure cultures were maintained on Wakimoto slants.

Pathogenicity test

Isolates collected from leaves of acid lime were tested for pathogenicity through detached leaf assay. In this process, firstly sterilized young leaves of acid lime were taken in a moist chamber and inoculated with bacterial suspension. The moist chamber was then incubated at 28$\pm$2^{0}C in the BOD incubator. Symptoms were developed after 3 weeks. Observations were made for the development of symptoms of bacterial spot (Fig.1)

Morphological and biochemical characterization of Xac

All the isolates of *Xanthomonas axonopodis* pv.*citri* were studied for typical morphological characters viz., colony characters, cell shape & size. All the isolated strains were identified and characterized biochemically by performing various standard biochemical tests viz; Gram staining reaction, KOH 3% test, Gelatine liquefication, Starch hydrolysis, Nitrate reduction, Citrate utilization, Oxidase test and Catalase tests.

DNA Isolation

DNA extraction of the bacterial isolates was carried out by CTAB method (Murray and Thompson, 1980). The DNA pellet obtained was resuspended in TE buffer and quantified using spectrophotometer and electrophoresed on 1% agarose gel.

Oligonucleotide primers for rep-PCR

Genetic diversity analysis of *X. Axonopodis* pv.*citri* isolates were evaluated by repetitive sequence based PCR (rep-PCR).Polymerase chain reaction (PCR) targeting repetitive DNA sequences (rep-PCR) such as repetitive extragenic palindromic sequences (REP), enterobacterial repetitive intergenic consensus sequences (ERIC) and BOX-PCR based on primers targeting the highly conserved repetitive DNA sequences of the Box A subunit of the BOX element were used for PCR amplification. The primer sequences are given below:

S. No.	Primer	Sequence (5' → 3')
1.	REP-PCR	
	REP1(reverse)	IIIICGICGICATCIGGC
	REP2(forward)	ICGICTTATTATCIGGCCAC
2.	ERIC-PCR	
	ERIC1(reverse)	ATGTAAGCTCCTGGGGATTCAC
	ERIC2(forward)	AAGTAAGTGACTGGGGTGAGCG
3.	BOX-PCR	CTACGGCAAGGCGACGCTGACG

PCR amplification conditions

The rep-PCR was performed in the thermocycler (BIORAD, C1000^{TM} Thermal cycle), under the conditions with REP primers as, REP1 and REP2, amplification began with a denaturation step at 95 °C for 7 min, followed by 35 cycles of 94 °C /1 min; 44 °C /8 min, and 65 °C for 8 min. With ERIC and BOX primers, cycling programmes started with denaturation at 95 °C for 7 min, followed by 30 cycles of: 94 °C/1 min; 1 min at 52 °C (ERIC) or 53 ºC (BOX) for primer annealing, and 8 min at 65 °C for primer extension. After the extension step, the temperature was maintained at 65 °C for 15 min for all three primer sets. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

Cluster analysis

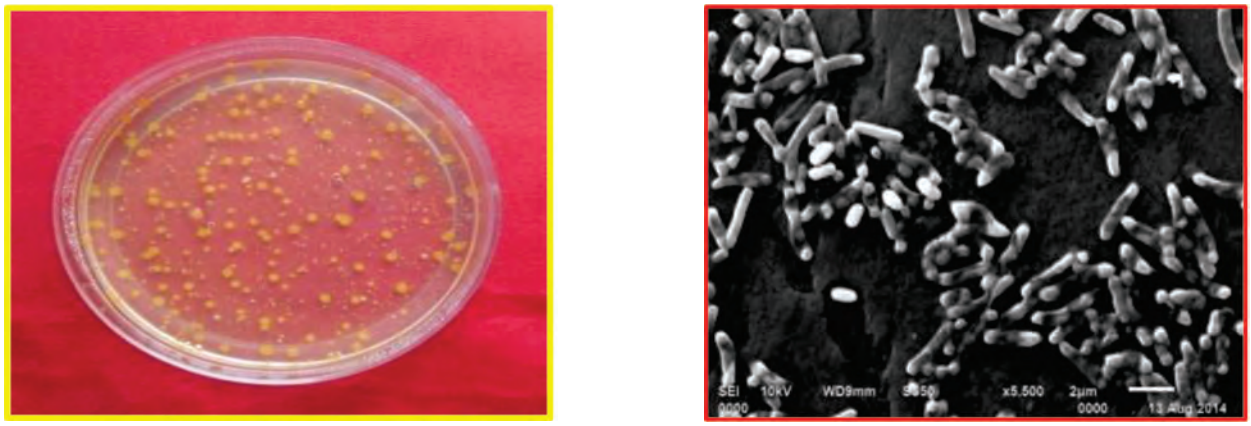
rep-PCR generated banding patterns of each isolate which were compared visually and grouped according to unique banding pattern. Each unique fingerprint generated by the PCR techniques was regarded as a haplotype. For the technique the representative isolates of a halophyte and isolates with unique banding pattern were electrophoresed on the same gel to confirm band identities and differences. Pattern was converted into binary data, i.e., the amplified products were scored for the presence "1" and absence "0", at each position along the lane.The genetic associations between isolates were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers.

$$\text{Similarity coefficient} = \frac{\text{Number of polymorphic bands}}{\text{Total no. of bands}}$$

The similarity matrix was subjected to cluster analysis by un-weighted pair group method for arithmetic mean (UPGMA) (Sneath and Sokal, 1973). Dendrogram was generated with the SHAN (Sequencial, agglomerative, hierarchical, and nested clustring methods) using the UPGMA method. The computations were performed

In vitro pathogenicity test -Development of corky lesion, one week after inoculation

Fig. 1. Pathogenicity Test



Bacterial colonies in Wokimoto isolates under SEM **Rod shaped cells observed by all the medium**

Fig. 2. Morphological characteristics

using the program NTSYS – PC (version 2.11s; Rohlf, 2003).

RESULTS AND DISCUSSION

Morphological and biochemical characterization of Xac

The Xac isolates were similar to one another in most of the morphological characters. All the isolates in the present studies were found to be short rod shape with rounded ends and polar flagella (Fig. 2). The size recorded was 2µm in length. In colony characters, all the isolates showed round, yellow and mucoid type of colony on Wakimoto medium (Fig. 2). According to Braithwaiten *et al*. (2002) *X. axonopodis* pv*. citri* colonies are straight, short rod-shaped measuring 1.5-2.0 x 0.5-0.75 µm. It has a predominantly single and polar flagellum. It produces yellow pigmented, mucoid colonies on agar plates with glucose or any other form of sugar. Presence of sugar makes the colonies very mucoid due to the production of extracellular polysaccharide slime. While the yellow colour of the colonies is the result of xanthomonadin pigment production. Das (2003) also reported the bacterium *X. axonopodis* pv*. citri* was rod-shaped measuring 1.5- 2.0 x 0.5- 0.75 µm, gram-negative and had a single polar flagellum with aerobic growth.

Biochemical tests are reliable and specific, and have been extensively used (add reference). All the isolates of *X. axonopodis* pv*. citri* were identified on the basis of certain biochemical tests. All the isolates were found positive for KOH (3%), gelatin liquification, starch hydrolysis, citrate utilization and catalase tests, while negative for gram staining, nitrate reduction, oxidase and asparagine tests (Fig. 3). Thus on the basis of these results, all the 13 isolates were considered to belong to genus *Xanthomonas*. The results in the present study are in accordance with Hsin-Cheng Lin *et al.* (2005) that all the *X. axonopodis* pv.*citri* isolates possessed hydrolysis ability of gelatine and were positive in utilization of different carbon sources.

Arshiya *et al*. (2014) isolated fifty six strains of *X. axonopodis* pv.*citri* from different citrus growing areas

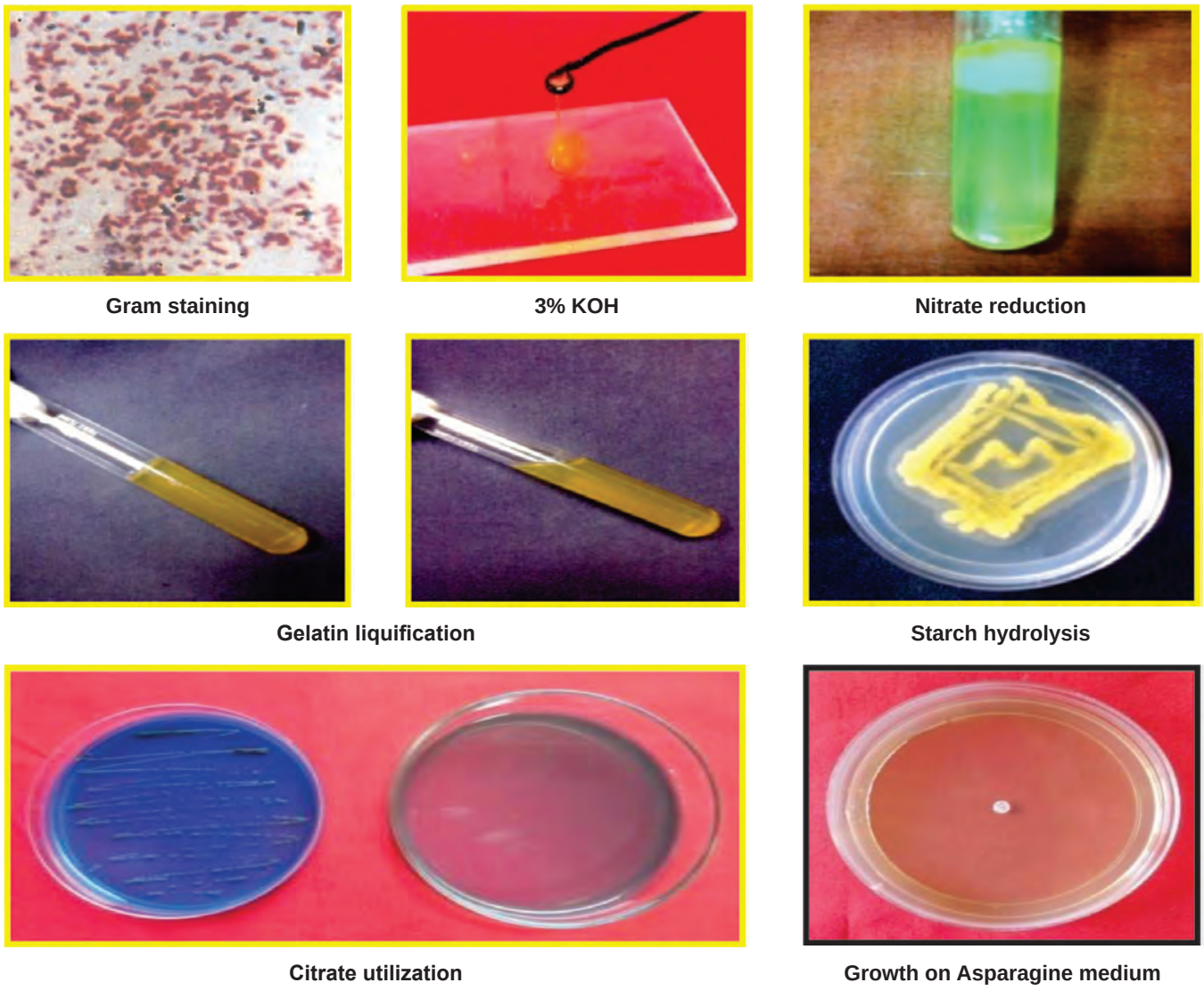


Fig. 3. Biochemical characteristics

of Marathwada region of Maharashtra state. They identified the pathogen biochemically by performing standard procedures given in the laboratory manuals by Goszczynska (2000), of biochemical tests like starch hydrolysis, gelatin liquefaction, aesculin hydrolysis, tween80 lypolysis, milk hydrolysis, oxidase test, H_2S production, urease production, and utilization of carbon from different sources including arabinose, fructose, sucrose and trehalose were used.

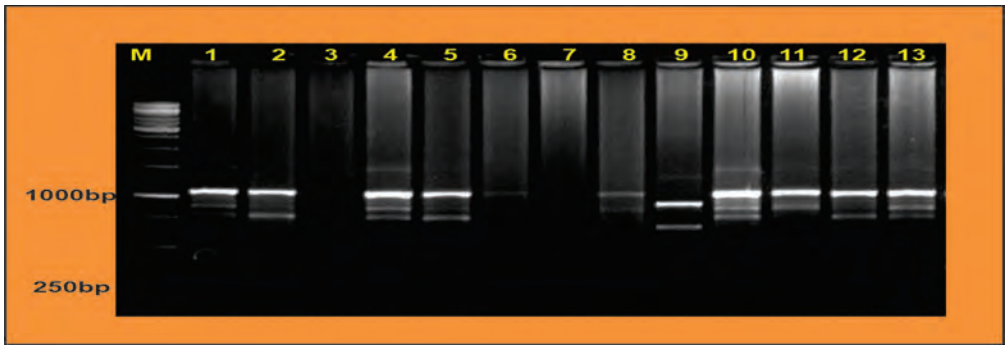
Genomic diversity analysis

rep- PCR were used to analyze the haplotypic distribution of isolates from different locations of Uttarakhand. All the 13 isolates differentiated into 13 distinct banding patterns (haplotypes) by rep-PCR technique.

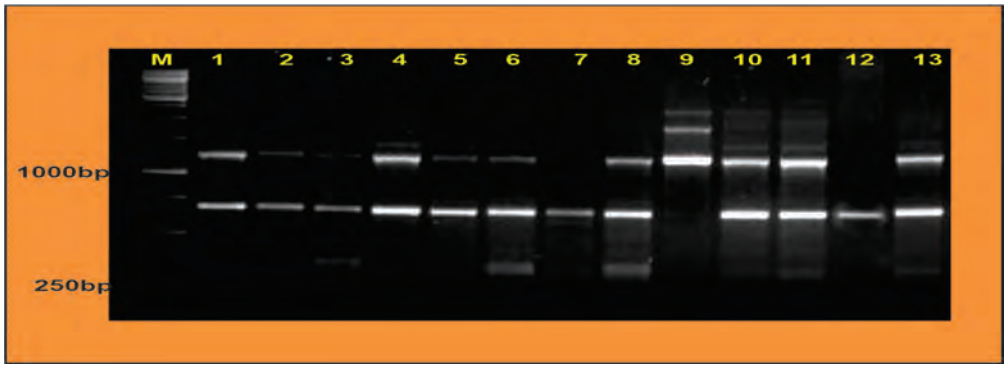
DNA fingerprinting of 13 isolates using PCR based assay

Electrophoretic profile with BOX primer

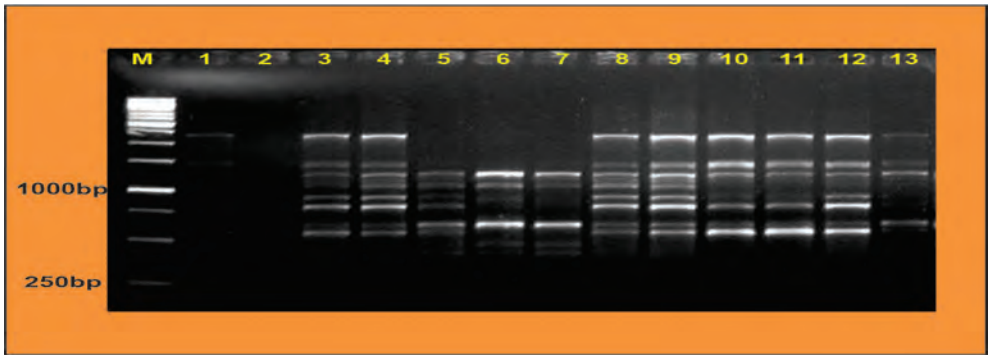
Box primer generated unique fingerprint pattern of haplotypes of *X. axonopodis* pv. *citri* isolates collected from thirteen locations. Box primer amplified a minimum of 0 and maximum of 4 bands for whole set of isolates. Six different band positions and a total of 27 bands were scored by Box primer. Some haplotypes had showed identical box fingerprints (lane 1, 4 and 10), thus similarity suggested that they had a common evolutionary history. Similarly three other haplotypes i.e lane 2, 5 and 12 are also similar. Haplotypes (lane 11, 12 and 13) shares nearly identical fingerprints but differs on the basis of two band positions (Fig. 4).



Electrophoretic profile with BOX primer



Electrophoretic profile with REP primer



Electrophoretic profile with ERIC primer

Fig. 4. Electrophoretic profile with BOX, REP and ERIC primers.

Electrophoretic profile with REP primer

The number of scorable bands amplified with REP primers ranged from a minimum of 1 and maximum of 3 bands for whole set of isolates. Six different band positions and a total of 34 bands were scored by rep primer. Haplotypes (lane 1, 2, 4 and 11) were found to be similar showing common evolutionary history. While the size and distribution of haplotypes (lane 3, 4, 5 and 8) were found to be highly similar with minor differences were observed, suggesting isolates were closely related. At 750bp position a band is present at all the isolates except in isolate 9 (Fig. 4).

Electrophoretic profile with ERIC primer

A total of 73 discrete bands were scored by ERIC primer with 10 different band positions. The amplification of genomic DNA of 13 *X. axonopodis* pv. *citri* isolates showed a minimum of 0 and maximum of 9 bands for whole set of isolates. Some haplotypes (lane 8 & 9 and 10 & 11) having multiple bands of equal mobility suggesting common evolutionary history. While some of the haplotypes had nearly identical fingerprints (lane 3, 4, 10, 11 and 12) with minor difference in some band positions (Fig. 4).

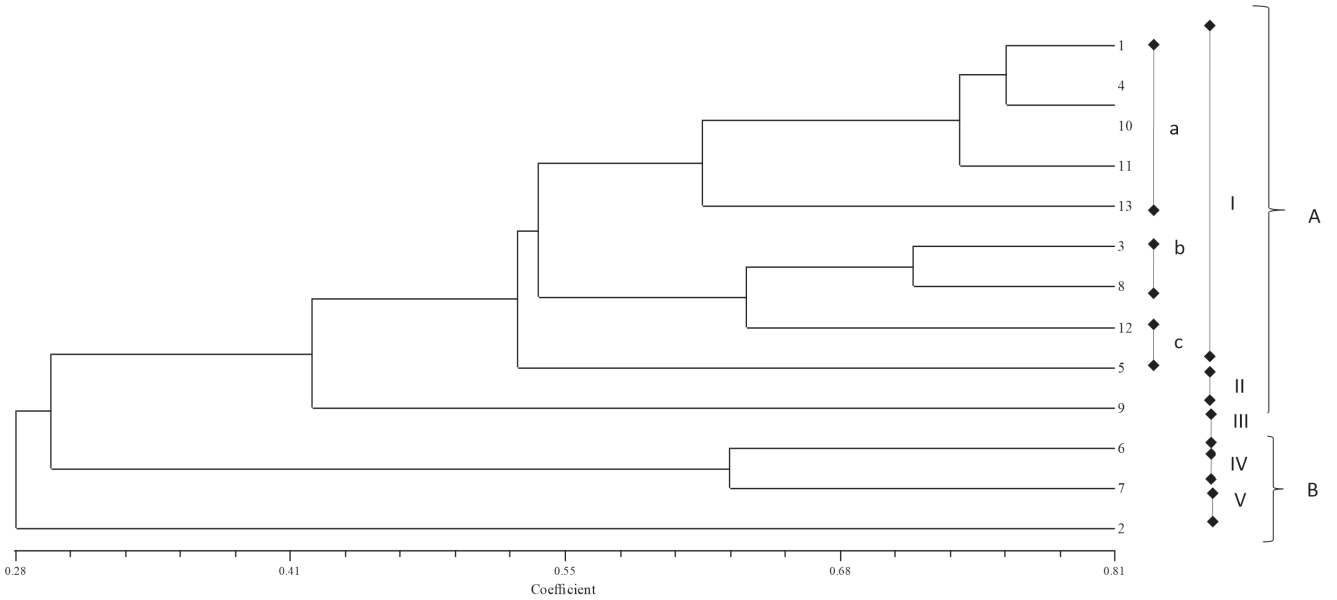


Fig. 5. Dendrogram of 13 isolates of *Xanthomonas axonopodis* pv. *citri* constructed by UPMGA based on Jaccard's coefficient

Location of the isolates collected:
1-Pantnagar, 2-Dehradun, 3-Haldwani, 4-Nagla, 5-Kicha, 6-Rudrapur, 7-Horticultural Research centre (Patharchatta), 8-Lalkuan, 9-Mota Haldu, 10-Jawahar nagar, 11-Kathgodam, 12-Golapar-I, 13-Galapar-I

Combined cluster analysis

UPGMA cluster analysis of the fingerprints generated by BOX, REP AND ERIC primers revealed considerable diversity among the isolates collected from different areas of Uttarakhand, presented in dendrogram (Fig. 5).Cluster analysis grouped the isolates into two main clusters (A & B) with 28 per cent similarity. The similarity co-efficient ranged from 0.058 to 0.81 indicating a good level of divergence. Grouping of the 13 isolates on the basis of SSR polymorphism resulted in two main clusters comprising of 10 and 3 isolate in each cluster with less than 65 per cent similarity. Isolate-2 (Dehradun) formed a divergent group from rest of the isolates. Whereas, isolate-4 and isolate-10 were found have maximum similarity (81 %).

The main cluster A is further divided into three sub clusters *viz.,* I, II, and III with 8, 1 and 1 isolates in each cluster. Sub cluster I being the largest sub cluster further had three groups *viz.,* group '*a'* with Isolate-1, Isolate-4, Isolate-10 and Isolate-11 followed by group '*b*' with Isolate-13 isolate and '*c*' was having 3 isolates *viz.,*Isolate-3, 8 and 12. Cluster II and III under main cluster A were the two divergent groups with Isolate-5 and Isolate-9 isolate respectively.

The Main cluster B contains two sub cluster such as IV and V with 2 and 1 isolate in each. Sub cluster IV found to have 2 isolates *viz.,* isolate-6 and 7 with 65 per cent similarity. The sub cluster V was the most divergent group containing isolate-2.

Similarity coefficient

Data scored from 13 isolates of *X. axonopodis* pv. *citri* with three primers were used to generate similarity coefficients. The pair wise similarities among 13 isolates were shown in Table1. The similarity co-efficient ranged from 0.058 to 0.813. The result of pair wise combinations indicated highest similarity between isolate - 4 & 10 with 0.813 similarity co-efficient. While the lowest similarity co-efficient was 0.058 between isolate-2 and isolate-9 indicating that these two are the most diverse isolates.

Thus, the results on molecular characterization revealed that the isolates collected from different locations of Uttarakhand were found diversified; it may be due to different geographic location of isolates. The present investigation is in accordance with Arshiya *et al*. (2014) who isolated different *X. axonopodis* pv. *citri* isolates from Maharashtra state, they separated two diverse groups within species of *X. axonopodis* pv. *citri* based on the geographic region of isolation through Cluster analysis by combining the banding patterns of REP-PCR, ERIC-PCR and BOX-PCR. Similar study was also done by several workers; Rezaei *et al.* (2012) collected and isolated twenty-five strains from provinces of Iran and assessed them phenotypically and genetically. Hsin-Cheng Lin, *et al.* (2005) studied the genetic diversity among the 46 strains of *X. axonopodis* pv. *citri* from Taiwan and analyzed by REP- and ERIC-PCR.

Table 1. Similarity matrix for Jaccard's coefficient

S.No.	Isolate-1	Isolate-2	Isolate-3	Isolate-4	Isolate-5	Isolate-6	Isolate-7	Isolate-8	Isolate-9	Isolate-10	Isolate-11	Isolate-12	Isolate-13
Isolate-1	1.000												
Isolate-2	0.455	1.000											
Isolate-3	0.400	0.153	1.000										
Isolate-4	0.733	0.333	0.563	1.000									
Isolate-5	0.428	0.400	0.461	0.600	1.000								
Isolate-6	0.188	0.300	0.384	0.352	0.546	1.000							
Isolate-7	0.067	0.111	0.250	0.250	0.400	0.625	1.000						
Isolate-8	0.389	0.188	0.714	0.611	0.533	0.467	0.267	1.000					
Isolate-9	0.263	0.058	0.533	0.473	0.375	0.235	0.200	0.588	1.000				
Isolate-10	0.785	0.357	0.500	0.813	0.533	0.294	0.188	0.473	0.421	1.000			
Isolate-11	0.692	0.333	0.615	0.733	0.538	0.357	0.230	0.563	0.412	0.785	1.000		
Isolate-12	0.500	0.255	0.667	0.667	0.583	0.285	0.250	0.600	0.438	0.600	0.615	1.000	
Isolate-13	0.538	0.400	0.461	0.600	0.500	0.417	0.272	0.438	0.294	0.642	0.667	0.583	1.000

Authors' contribution

Conceptualization of research work and designing of experiments (AN, JS, PK); Execution of field/lab experiments and data collection (AN, JS, PK); Analysis of data and interpretation (AN, JS); Preparation of manuscript (AN, JS, PK)

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