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Genetic Variability within *Xanthomonas axonopodis pv. punicae*, Causative Agent of Oily Spot Disease of Pomegranate

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Abstract

Bacterial blight is one of the most devastating diseases of pomegranate in India. It is known to be caused by the strains of *Xanthomonas axonopodis pv. punicae (xap)*. As the control of disease varies with the use of pesticide agents, it becomes imperative to study the genetic variation among the pathogenic strains. Thirty-six strains of *Xanthomonas axonopodis* pv. *punicae*, were isolated from the diseased fruits of 3 varieties of pomegranate originating from 3 different provinces of Maharashtra, India. All the strains characterized phenotypically and genotypically were found to have diversity. The genetic diversity among the 36 Xanthomonas isolates was assessed using RAPD based techniques. A cluster dendrogram based on the random amplified polymorphic DNA (RAPD) showed that genetic diversity existed among the isolates of Xanthomonas. The genomic variation was found to be in the range of 0.55% to 0.95% among the isolates. The cluster analysis based upon band patterns formed two major clusters with 4 sub-groups. It can be concluded that this variability was genetic in nature irrespective of their location and the variety of pomegranate.

Keywords: Bacterial blight; *Xanthomonas axonopodis* pv. *punicae*; Genetic variability; RAPD

Introduction

Pomegranate is one of the most economically important fruit crops of India. Maharashtra region is the largest producer of pomegranate in the country. Pomegranate production suffers severely by the disease called oily spot disease which is predominant in the pomegranate cultivation in India. The quality and productivity of pomegranate crop is hampered by 70% to 80% due to this disease [1,2]. The bacterial blight was first reported in India from Rajasthan in 1952 [3]. It received a minor importance earlier but appeared as a serious threat in all pomegranate growing regions of Maharashtra, Northern Karnataka and Andhra Pradesh [4]. Solapur, Sangli, Nasik, Satara, Pune, Ahmadnagar and Wardha districts of Maharashtra state are major cultivators of Pomegranates, with minor plantations in other areas [5]. Since 1998, Oily spot disease has appeared as a major production problem in important pomegranate growing states in India [6]. The oily spot disease was first observed in the Maharashtra state at Mohol village of Pandharpur area in Solapur district in 2003 [7]. The disease continued to damage the crop for subsequent years, inspite of all possible and available protection measures adopted by the farmers. The disease could not be mitigated effectively due to rapid buildup of inoculums and spread of the disease widely. Many measures like biocontrol agents, combinations of antibiotics and pesticides along with phytochemicals and cultivation practices were not sufficient to remedy the problem [8,9]. There are reports of considerable amount of strain variation in the strains of *Xanthomonas axonopodis* pv. *punicae* from different parts of Karnataka [10]. Earlier reports based on physiological and biochemical tests have been used for characterization and study of phenotypic variation among *Xanthomonas* species [11-14] which are still much needed for identification of plant pathogenic bacteria to genus and species level [15]. Modern genetic techniques allow the exact differentiation of genetic variation within a population [16]. A rapid and specific identification test would be very useful to monitor the infection of pomegranate plants in order to develop strategies to control the disease in fields. A genetic variability study is essential to enhance the understanding of its taxonomy, epidemiology, and identification of the bacterial strain [17]. The similar control measures are not found to be very effective on all the varieties and their growing locations of pomegranate due to the existence of genetic variation among the strains responsible for the disease. Considering the seriousness of the disease, the present study was carried out to understand the diversity in *Xanthomonas axonopodis* pv. *punicae* prevalent among different pomegranate varieties and their various locations of Maharashtra in India.

Materials and Methods

Collection of diseased pomegranate fruit samples and isolation of organisms

Diseased fruit samples belonging to oily spot disease affected 3 varieties of pomegranate (Bhagawa, Ganesh and Mridula) were collected separately from 3 districts viz. Sangli, Solapur and Nasik of Maharashtra state. The basis used for sampling was the above three varieties are popular in the above mentioned three districts of Maharashtra. All the samples were carried to the laboratory in sterile polythene bags and stored at 4°C.

The infected fruit samples showing typical symptoms of bacterial blight were taken up for the isolation of the causal agent. The fruit samples were washed thoroughly with tap water and allowed to dry. The samples were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for one minute and washed three times serially in sterile distilled water to remove the traces of mercuric chloride. The infected portion was cut into small pieces and suspended in 10 mL of sterilized distilled water and squeezed gently with sterilized scalpel. When the water became slightly turbid due to bacterial cells, the suspension was serially diluted

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up to 10^6 dilutions in 9 mL sterile water blanks. 0.1 mL of diluted bacterial cell suspension was spread on the sterilized nutrient agar petri plates. The inoculated plates were incubated at 30°C for 72 hours. The plates were observed for the development of well separated, typical, light yellow coloured bacterial colonies resembling *Xanthomonas* sp. Four colonies were chosen from each plate and purified by four quadrant streak method. The 36 bacterial isolates obtained upon isolation from the diseased fruit samples were designated as Xa1 to Xa36 and were stored at -80°C in glycerol stocks [15].

Morphological and biochemical characterization of the isolates for identification

The colony growth and morphological characteristics of the pathogen such as cell shape, size, color, Gram reaction characters were studied [15]. The biochemical characters such as hydrolysis of starch, gelatin liquefaction, hydrogen sulphide production, catalase, oxidase and acid production from different sugars *viz*., glucose, lactose, fructose, sucrose, mannitol, maltose and dextrose by the isolates were studied [15,18,19]. The isolates were identified by comparing the characteristics with their description reported in Bergey's Manual of Systematic Bacteriology [19,20].

Pathogenicity testing

The freshly collected healthy pomegranate samples were used for pathogenicity test. The fruits samples were washed three times with sterilized water followed by surface sterilization with 10% sodium hypochlorite wash for 1 minute. The traces of sodium hypochlorite were removed by sterile water wash. Pomegranate samples were pricked by a sterile needle and sprayed with cell suspension of *Xanthomonas axonopodis* isolates as per their same fruit variety as well as other fruit varieties for cross infectivity (2×10^8 cfu/mL). In cross infectivity studies the isolates of Bhagawa variety were used to infect fruits of Ganesh and Mridula variety collected from same and different location. Likewise, the cross-infectivity studies were carried out with isolates from Ganesh and Mridula varieties. The inoculated fruits placed in sterile beakers, packed with sterile polythene bags for avoiding cross infectivity, were incubated at room temperature at the normal light. They were observed periodically for 4 weeks for the development of disease symptoms. The pathogens were reisolated from the diseased fruits and compared with their original cultures for their verification.

Susceptibility test of the isolates against broad spectrum antibiotics

Antibiotic susceptibility test was performed by agar well diffusion assay with all the 36 pathogenic isolates with 2.5 mg/mL of streptomycin sulfate (HiMedia) and Streptocycline (a broad-spectrum systemic antibacterial antibiotic product from Hindustan Antibiotics Ltd. 90% streptomycin sulfate + 10% tetracycline hydrochloride). The zones of inhibition were recorded.

Genetic variability

Extraction of total genomic DNA-The total genomic DNA was isolated for PCR-RAPD: Pure culture of each strain was streaked on the nutrient agar plates and incubated at 30°C for 72 h. Single colony from each plate was inoculated in 10 mL nutrient broth contained in 100 mL conical flasks. The flasks were incubated at 30°C for 72 h in shaker incubator at a speed of 120 rpm. About 1.5 mL aliquots of broth from each flask were taken in 2.5 mL Eppendorf tubes and centrifuged at 13000 rpm for 5 min. The DNA extraction was followed as per the method of Yenjerappa [21]. The supernatant was poured off, 200 µL of lysis buffer was added to the tubes containing sediment pellet and mixed well. 166 µL 5M NaCl was added and vortexed for thorough mixing, the contents were again centrifuged at 13000 rpm for 10 min. The obtained supernatant was collected in a fresh tube to which 1 µL RNase A (10 mg/mL) was added, mixed well and incubated at 37°C for 30 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed gently by inverting the tubes and centrifuged at 13000 rpm for 6 min. The upper aqueous phase was transferred to a fresh clean tube; 1.0 mL of cold 95% ethanol was added and mixed gently. The tubes were kept in deep freezer at -20°C for 1hr and centrifuged at 13000 rpm for 6 min. Ethanol was poured off, DNA pellet was air dried using speed vacuum for 5 min. The pellet was resuspended in 50 µL of 1X TE buffer, kept in the refrigerator at 4°C for overnight and stored in deep freezer at -20°C. The DNA was quantified using a spectrophotometer and electrophoresed on 0.8% agarose gel by comparison with control DNA samples of known concentration. The extracted DNA samples were taken up for RAPD analysis.

RAPD analysis

The total 10 random primers (OPA, OPB and OPF) were used in this study. The RAPD analysis was performed as per the procedure reported earlier with some modifications [22]. The PCR mixture was consisted of 2.5 µL 10X assay buffer with 15 mM $MgCl_2$, 1.0 µL dNTPs mix (2.5 mM each), 1.0 µL primer (5 pM μL^{-1}), 1.0 µL template DNA (25 ng μL^{-1}), 14.30 µL sterile nuclease free water and 2.0 µL Taq DNA polymerase (3.0 U μl^{-1}) (M/s Banglore Genei, Pvt. Ltd., Banglore, India).

The PCR amplifications were performed with Thermal Cycler (Thermo). The program was set for initial denaturation at 94°C for 4 min followed by 40 cycles at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min. The PCR products were analyzed by 0.8% agarose gel electrophoresis in 1X TAE buffer and stained with Ethidium bromide (0.5 µg mL^{-1}). The gel was visualized under UV transilluminator. The amplification patterns of all the isolates were compared with each other and bands of DNA fragment scored as '1' for presence and '0' for absence, generating '0' and '1' matrix. The DICE coefficient was used for the estimation of genetic variability. Pair-wise genetic similarities between strains were estimated by similarity coefficient. Clustering was done using the similarity coefficient and cluster obtained based on unweighted pair group method with arithmetic averaging (UPGMA) using cluster analysis of PyElph 1.4 PC software [23].

Results and Discussion

A total 36 (Xa1-Xa36) isolates causing oily spot disease of pomegranate were successfully isolated from diseased fruit samples of Bhagawa, Ganesh and Mridula varieties located in the pomegranate growing areas of Sangli, Solapur and Nasik districts of Maharashtra, India (Table 1). The isolated colonies emerged as shining yellow and mucoid on nutrient agar. They were Gram negative in nature and resembling with the *X. axonopodis* LMG 859 (the reference strain of *X. axonopodis* pv. *punicae* obtained from Shivaji university Kolhapur as a positive control). These isolates identified as *X. axonopodis* pv. *punicae*

Location	Sangli			Solapur			Nasik		
Variety	Bhagawa	Ganesh	Mridula	Bhagawa	Ganesh	Mridula	Bhagawa	Ganesh	Mridula
No. of Isolates	4	4	4	4	4	4	4	4	4

Table 1: The location of collection and variety of diseased fruits with the number of pathogenic isolates.

Isolate no.	Time in Days	Isolate no.	Time in Days
Bhagawa (Sangli)		**Mridula (Solapur)**	
Xa1	15	Xa21	16
Xa2	18	Xa22	22
Xa3	22	Xa23	21
Xa4	17	Xa24	22
Ganesh (Sangli)		**Bhagawa (Nasik)**	
Xa5	17	Xa25	17
Xa6	14	Xa26	17
Xa7	20	Xa27	19
Xa8	21	Xa28	20
Mridula (Sangli)		**Ganesh (Nasik)**	
Xa9	20	Xa29	20
Xa10	22	Xa30	22
Xa11	23	Xa31	18
Xa12	19	Xa32	21
Bhagawa(Solapur)		**Mridula (Nasik)**	
Xa13	23	Xa33	22
Xa14	24	Xa34	18
Xa15	20	Xa35	23
Xa16	23	Xa36	16
Ganesh (Solapur)			
Xa17	23		
Xa18	18		
Xa19	19		
Xa20	24		

Table 2: Oily spot disease causing isolates with time required for the complete development of disease.

Sr. No	Strain No.	Size of zone of inhibition	
		Streptomycin Sulfate	Streptocycline
1	Xa1	9	15
2	Xa2	12	21
3	Xa3	6	7
4	Xa4	0	9
5	Xa5	8	6
6	Xa6	16	16
7	Xa7	6	9
8	Xa8	0	6
9	Xa9	17	18
10	Xa10	6	7
11	Xa11	8	10
12	Xa12	15	16
13	Xa13	0	20
14	Xa14	7	8
15	Xa15	16	16
16	Xa16	6	6
17	Xa17	14	16
18	Xa18	10	12
19	Xa19	10	14
20	Xa20	15	6
21	Xa21	0	8
22	Xa22	8	20
23	Xa23	10	6
24	Xa24	6	13
25	Xa25	15	18
26	Xa26	8	9
27	Xa27	6	12
28	Xa28	12	6
29	Xa29	15	16
30	Xa30	8	10
31	Xa31	0	6
32	Xa32	6	19
33	Xa33	16	9
34	Xa34	9	14
35	Xa35	0	6
36	Xa36	0	10

Table 3: Inhibitory pattern of the isolates against wide spectrum antibiotics.

based on their cultural, morphological and biochemical characteristics [19,20].

All the 36 strains of *X. axonopodis* were found to be positive during *in vivo* pathogenicity test on fresh and healthy fruits as they displayed the symptoms similar to oily spot disease. All the strains were successfully reisolated from the infected fruits and found to be matching their parent strains. The variations were observed in the infectivity and pathogenicity pattern during *in vivo* pathogenicity tests. The difference was observed in time required for the complete development of disease. The time required for the development of disease was varied from 14 to 24 days with all the 36 isolates (Table 2). Some isolates infected immensely and exhibited early symptoms of the oily spot disease, the others were found to infect very slowly resulting in delayed appearance of disease symptoms.

Biochemical variability

A wide diversity was observed among the strains for the following biochemical tests:

Starch hydrolysis: Out of 36 all the 29 strains hydrolyzed starch except the 7 (Xa2, Xa5, Xa19, Xa24, Xa26, Xa29 and Xa36). The strains which were positive in starch hydrolysis varied in their degree of hydrolysis. The strains isolated from Ganesh variety, Xa6 (Sangli), Xa18 (Solapur) and Xa30 (Nasik) exhibited maximum zone of starch hydrolysis among all the 36 isolates.

Gelatin liquefaction: All the 36 strains found to be positive for gelatin liquefaction test.

Hydrogen sulfide production: Out of 36 strains 30 were positive for H_2S gas production test only 6 strains were not observed to be negative (Xa1, Xa17, Xa20, Xa23, Xa25, Xa35) showing variation in their ability to produce H_2S.

Catalase: All the 36 strains showed catalase test positive.

Oxidase: Out of 36, 27 strains showed positive to oxidase and 9 were negative.

Acids from carbohydrate: Except Xa2 and Xa26 all other strains produced acids from glucose as a carbon sources. When fructose was used as a carbon source all the 36 strains produced acid by utilizing it. When lactose, maltose and mannitol used as a carbon sources all the strains failed to produce acids. In case of sucrose except Xa6, Xa12, Xa16, Xa17, Xa21, Xa30, Xa33 all other strains utilized sucrose as a carbon source and produced acids. The results clearly indicate the variation in utilization of carbohydrate and production of acid.

There was no relation between the biochemical characteristics and disease progress or infectivity pattern during pathogenicity studies. Giri et al. was also recorded the biochemical variability within same species but not found any correlation with disease progress [10].

Susceptibility test

The strains were found to differ in susceptibility test against

different wide spectrum antibiotics such as streptomycin sulfate and Streptocycline (Table 3). Due to their variation in susceptibility test against wide spectrum antibiotics all the strains were taken up for determining their genetic variations.

The results from the 36 *Xanthomonas axonopodis* pv. *punicae* strains isolated from infected fruits of pomegranate showed variations in biochemical tests and susceptibility test against the wide spectrum antibiotics.

Molecular variability

Genomic DNA extraction of Xanthomonas isolates: Thirty six isolates of *Xanthomonas axonopodis* causing oily spot disease were subjected to DNA extraction and the DNA samples were run on 0.8% agarose gel electrophoresis to check the integrity of DNA by observing under UV transilluminator.

RAPD analysis: Due to the existence of remarkable variation in susceptibility and pathogenicity the genetic diversity was assessed between *Xanthomonas axonopodis* isolates through RAPD. Main advantages of the RAPD technology include (i) suitability for work on anonymous genomes, (ii) applicability to problems where only limited quantities of DNA are available, (iii) high efficiency at low expense [24]. RAPD is the ideal technique in the absence of complete sequence information about genome of the pathogenic isolates, since it scans for sequence variation throughout the whole genome. The random amplification of polymorphic DNA (RAPD) is a quick method for developing species-specific probes and primers [25]. Molecular genetic markers have been developed into powerful tools to analyze genetic relationships and genetic diversity among various microbial groups. As an extension to the variety of existing techniques using polymorphic DNA markers, RAPD technique may be used in molecular ecology to determine taxonomic identity, assess kinship relationships, analyze mixed genome samples, and create specific probes.

Primer screening for RAPD analysis: Ten different decamer primers were screened out as mentioned in Table 4. The primers were diluted up to 10 pMole concentration with nuclease free water. During the RAPD analysis the amplification could be achieved only with two primers from operon primers series i.e. OPA-02 and OPB-03. Different annealing temperature conditions were checked across the set of Ten decamers i.e. 25°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C and 38°C, the optimum annealing temperature was found to be 35°C for all the decamers used in the present study.

Amplification and dendrogram observed by decamer OPA-02: In RAPD analysis the amplification was observed using OPA-02. Other primers used in this study could not be amplified with all the 36 isolates of *Xanthomonas axonopodis*. The other primers were not amplified in all the isolates and banding pattern was not observed. Only OPA-02 primer exhibited good amplification with scorable bands. The amplification was observed in size range of 300 bp to 1800 bp.

Sr. No	Primer	Sequences
1	OPA-02	5'- TGC CGA GCT G3'
2	OPA-03	5'- AGT CAG CCA C 3'
3	OPA-08	5'- GTG ACG TAG G3'
4	OPA-20	5'- TCG GCG ATA G3'
5	OPB-02	5'- TGA TCC CTG G3'
6	OPB-03	5'- CAT CCC CCT G3'
7	OPB-04	5'- GGA CTG GAG T3'
8	OPB-05	5'- TGC GCC CTT C3'
9	OPF-06	5'- GGG AAT TCG G3'
10	OPF-07	5'- CCG ATA TCC C3'

Table 4: Random primers used for the amplification of *X. axonopodis pv. punicae* DNA.

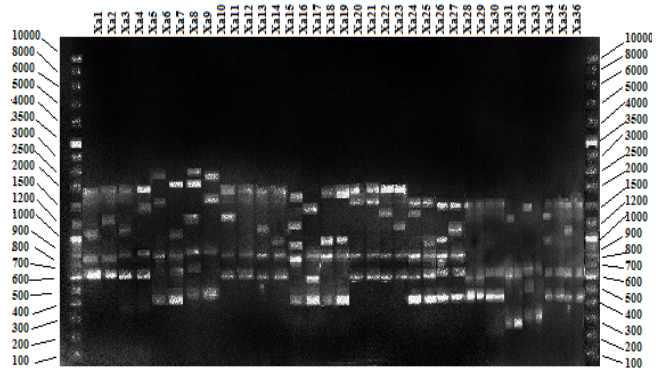

Figure 1: RAPD band pattern of amplified *Xanthomonas axonopodis pv. punicae* isolates.

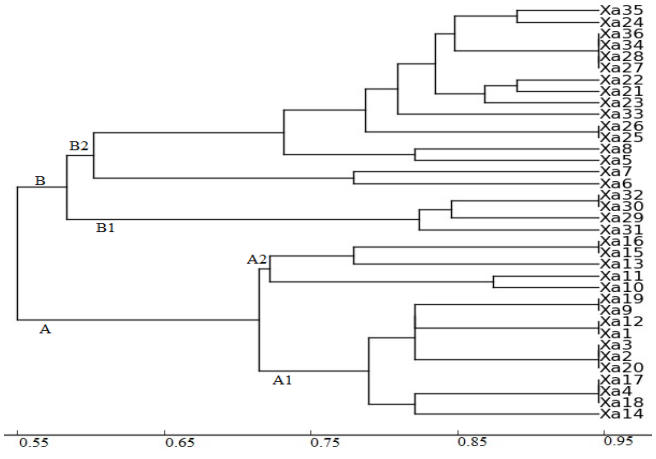

Figure 2: The dendrogram generated based on the RAPD data using the unweighted pair group method with arithmetic mean cluster analysis of genetic similarity coefficients.

Genetic variability among 36 isolates of *Xanthomonas axonopodis* pv. *punicae* was determined through RAPD analysis by using random primer OPA-02. The analysis resulted into 140 monomorphic and polymorphic bands. It revealed that maximum isolates exhibited monomorphic bands at 450, 650, 750 bp and polymorphic bands at 400, 900, 1000, 1100, 1500, 1700 bp (Figure 1). In OPA-02 primer *Xanthomonas axonopodis* pv. *punicae* showed 100% polymorphism. The dendrogram constructed based on unweighted pair group method with arithmetic averaging (UPGMA) analysis formed amplification with primer OPA-02 was comprised of two clusters A and B with a genetic similarity coefficient 0.58 (Figure 2 and Table 5). Cluster A and B were further divided in to two different sub-clusters A1, A2 and B1, B2. From these two clusters A and B (Xa17, Xa4, Xa18), (Xa19, Xa9), (Xa15, Xa16), (Xa30, Xa32), (Xa25, Xa26), (Xa27, Xa28, Xa34, Xa36) were very close with similarity coefficient ranged from 0.71 to 0.85. These isolates exhibited very minor variation so they grouped in the same clusters.

The Strains observed in cluster A were isolated from Bhagawa, Ganesh and Mridula varieties collected from Sangli and Solapur. The Strains observed in cluster B were also isolated from Bhagawa, Ganesh

Variety and Location of Pomegranate	Cluster A		Cluster B	
	A1	A2	B1	B2
Sangli				
Bhagawa	Xa1, Xa2, Xa3, Xa4	_	_	_
Ganesh	_	_	_	Xa5, Xa6, Xa7 Xa8
Mridula	Xa9, Xa12	Xa10, Xa11	_	_
Solapur				
Bhagawa	Xa14	Xa13, Xa15, Xa16	_	_
Ganesh	Xa17, Xa18, Xa19, Xa20	_	_	_
Mridula	_	_	_	Xa21, Xa22, Xa23, Xa24
Nasik				
Bhagawa	_	_	_	Xa25, Xa26, Xa27, Xa28
Ganesh	_	_	Xa29, Xa30, Xa31, Xa32	_
Mridula	_	_	_	Xa33, Xa34, Xa35, Xa36

Table 5: Grouping of 36 pathogenic isolates in different clusters on the basis of cluster analysis.

and Mridula varieties collected from Sangli, Solapur and Nasik. In the cluster A strains isolated from Ganesh, Bhagawa and Mridula varieties (Xa17, Xa4, Xa18), (Xa3, Xa2, Xa20), (Xa15, Xa16), (Xa1, Xa12), (Xa9, Xa19) with locations Sangli and Solapur districts exhibited very close relationship with each other. Similarly, in the cluster B strains isolated from above three varieties (Xa30, Xa32), (Xa26, Xa25) and (Xa27, Xa28, Xa34, Xa36) with location Nasik district revealed a very close relationship.

In cluster A none of the strain found to be present from Nasik district. The strains isolated from different varieties of pomegranates but with the same region like Bhagawa (Xa1 to Xa4), Ganesh (Xa5 to Xa8), Mridula (Xa9 to Xa12) from Sangli and Bhagawa (Xa13 to Xa16), Ganesh (Xa17 to Xa20), Mridula (Xa21 to Xa24) from Solapur, formed different cluster and sub clusters showing polymorphism. As in cluster B strains isolated from Nasik with Bhagawa, Ganesh and Mridula varieties formed different sub clusters as per the varieties as Bhagawa (Xa30, Xa32) and Ganesh (Xa25, Xa26) exhibiting polymorphism. This revealed the genetic variation between the strains isolated from same location but different varieties. The strains Xa27, Xa28, Xa34 and Xa36 formed one sub cluster showing very close relationship with a similarity coefficient of 0.80 but these were isolated with Bhagawa and Ganesh varieties only from Nasik.

A similar difference was also present within the same variety and same location (Mridula from Sangli and Bhagawa from Solapur), this further affirmed the existence of variation. Study of RAPD pattern has already been reported for the rapid, sensitive, and specific detection of genetic diversity among species and strains of Streptomyces [26]. Variability within the same species, *X. axonopodis* has also been reported earlier for various other plant diseases [10,27,28]. Mondal and Mani have also reported the genetic variability among the *X. campestris* strains isolated from oily spot infected pomegranates through Maharashtra and Delhi using ERIC-PCR method [29]. The present study exhibited a wide genetic variation existed between the 36 *Xanthomonas axonopodis* isolates in the range of 0.55% to 0.95%.

It confirmed that the variation among the strains was irrespective of their location and variety of pomegranate (Figure 1). Similar kind of diversity had been reported earlier in *X. campestris* pv. *passiflorae* from southern Brazil [30]. Kishun and Gupta have a similar finding from *X. campestris* pv. *mangiferaeindicae* population with a significant level of genetic diversity and the formation of 2 clusters in the phylogenetic tree [31]. The results from this study showed a high rate of genetic diversity with a 0.95 similarity coefficient. This was in contrast to a earlier study by Odipio et al. [32], who found very low genetic diversity among Ugandan isolates of *X. campestris* pv. *musacearum* as determined by RAPD. This further confirms that a significant level of polymorphism was existing among the present 36 evaluated strains of *Xanthomonas axonopodis* pv. *punicae* [32].

The genetic diversity found among the 36 pathogenic isolates may developed due to the outcrossing behavior of pomegranate crop and the acquired antibiotic resistance due to overexposure to antimicrobial agents employed for the control of the disease.

It can be concluded that the biochemical, pathogenic, susceptibility and molecular techniques employed in the present study give strong evidence to the existence of variability among 36 *X. axonopodis* pv. *punicae* isolates regardless of variety and the geographical origin of oily spot disease in pomegranate. There is no correlation present between the biochemical, pathogenic, susceptibility and molecular variability patterns. The present study confirmed that the variability is existed between the 36 pathogenic isolates of *X. axonopodis* pv. *punicae*. This is the important finding providing information on the genetic structure of a pathogenic bacterial population. It might help in designing the strategy for the control of Oily spot disease of pomegranate.

Statistical analysis

The data was subjected to analysis of molecular variance (AMOVA) and the significance of the difference between the means was determined ($P<0.05$) using MedCalc. Values were expressed as means of 3 replicate determinations ± standard deviations (SD).

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