

Original Research Article

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Genetic Variability Amongst *Xanthomonas axonopodis* pv. *punicae* Studied by RAPD Banding Pattern Analysis

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ABSTRACT

Keywords

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Bacterial blight disease of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is one of the most destructive disease of pomegranate (*Punica granatum*) inflicting considerable quantitative and qualitative losses. Mostly the disease occurred on leaves, stems and fruits. The disease is characterized by small, irregular, translucent, water soaked spots with light to dark necrotic centre surrounded by prominent yellow margin on leaves. The isolates were made from the symptomatic samples collected from five different locations viz. Yekurga (Osmanabad), Killari (Latur), Kej (Beed), Nimgaon and Badnapur belonging to Jalna district of Maharashtra state, India. The primer OPA09, ABA05 and ABA07 was found most significant by producing 100 per cent polymorphism amongst 03 strains of Latur, Osmanabad and Beed districts, whereas, Primer OPC03, OPH02 and OPC20 was found most significant by producing 100, 100 and 90.91 per cent polymorphism amongst 02 strains of Jalna districts. These results indicate a high level of genomic variability among the isolates even within the different geographical regions.

Introduction

Pomegranate (*Punica granatum* L.) is a favourite table fruit in tropical and subtropical regions of the world which belongs to family *punicae*. It is native to Iran but extensively cultivated in Mediterranean regions especially in Spain, Morocco, Egypt and Afghanistan. Pomegranate is being cultivated on an area of 193.0 thousand ha in India with production of 2198.0 thousand metric tonnes and productivity of 11.3 metric tonnes per ha (Anonymous, 2016). The Maharashtra state, alone occupies an area of 128.65 thousand ha, followed by Karnataka (23.23 thousand ha.), Gujarat (14.77 thousand ha.) and Andhra Pradesh (5.38 thousand ha.). Even

Maharashtra is having largest area under pomegranate cultivation; production and productivity are 1197.71 thousand metric tonnes and 9.31 metric tonnes per ha, respectively (Anonymous, 2015). Incidence of bacterial blight disease of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap*) is the major obstacle and limiting factor in Maharashtra state which alone accounts for loss of cultivated area, particularly in Nasik, Solapur, Sangli and Ahmednagar districts. Bacterial blight infection results in appearance of water soaked oily spot symptoms on leaves, stems and fruits which consequently decreases fruit

production and market value. Severity of incidence and losses varies among different isolates and influenced by existing climatic conditions and geographical distribution (Mondal and Sharma, 2009; Mondal and Singh, 2009, Petersen *et al.*, 2010, Mondal *et al.*, 2012). The present investigation was carried out to understand molecular diversity in *Xanthomonas axonopodis* pv. *punicae* isolated from different parts of Jalna, Latur, Osmanabad and Beed district of Maharashtra state, India.

Materials and Methods

Collection of diseased sample and isolation of pathogen

The diseased leaves samples of Pomegranate showing typical symptoms of bacterial blight were collected for isolation of bacterium *Xanthomonas axonopodis* pv. *punicae* from five different locations *viz.* Yekurga (Osmanabad), Killari (Latur), Kej (Beed), Nimgaon and Badnapur (Jalna) belonging to different region of Maharashtra state during in the month of September and October, 2016. The isolation of bacterium was carried out by tissue isolation method on Nutrient Agar (NA) medium at 25 to 28 °C for 3-5 days. Welldeveloped, separated, typical, bright yellow, mucoid colonies on plate were further streaked onto the agar slants containing the NA medium and incubated at 25 to 28 °C for 3 days. Then cultures were stored in the refrigerator at 4 °C, which served as a stock culture for further studies. The isolates were designated as per different locations *viz.* Yekurga (*XapI*), Killari (*XapII*), Kej (*XapIII*), Nimgaon (*XapIV*) and Badnapur (*XapV*).

DNA isolation

DNA extraction from 5 samples of bacteria was carried out using SDS-Ammonium acetate method. Loop full of each culture was suspended in 0.2ml extraction buffer (50mM

Tris-HCl, 50mM EDTA, 250mM NaCl, 1.5%Sucrose) and 50µl of SDS (20%) was added to it. The solution was vortexed and incubated for 30 min at 65°C. 50µl of 7.5M Ammonium acetate was then added to the above solution and mixed by inverting. The tubes were incubated at 4°C for 15 minutes. DNA was extracted using 1 volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuged at 10000rpm for 5mins. Double volume of ethanol (96-100%) was added to the aqueous phase in a new tube, inverted twice and allowed to stand at 4°C for 30 minutes. The mixture was then centrifuged at 10000 rpm for 15 minutes. After drying for few seconds pellet was dissolved in 50µl elution buffer (10mM Tris-HCl, 1mM EDTA). Agarose gel electrophoresis was performed to check the presence of DNA using 0.8% Agarose. The DNA was stored at 4°C for further use.

DNA quantification

DNA concentration was determined using Quant-iT™dsDNA BR Assay Kit of Invitrogen (Table 1). Quant-iT™ working solution was prepared for 2 samples and 2 standards by diluting the Quant-iT™dsDNA BR reagent 1:200 in Quant-iT™dsDNA BR buffer in a plastic container. 190 µl of working solution was dispensed in 2 Qubit assay tubes for standards and 198 µl was dispensed in each sample tube. 10 µl of each of the two standards were added to the respective standard tubes and 2 µl of each of the sample was added to the respective sample tubes.

The tubes were vortexed and incubated at room temperature for 2 minutes. The Quant-iT™dsDNA BR was chosen on Qubit™fluorometer and calibration done using the two standards. Sample readings were taken and calculated for 2 µl. The results were obtained in µg/ml.

RAPD PCR protocol

The DNA isolated from five bacterial samples was subjected to polymerase chain reaction (PCR) amplification with 5 random 10-mer primers (Synthesized by GeNei, Bangalore, India) (Table 2). Amplification of genomic DNA was carried out in 25 µl reaction mixture containing 1µl (20 ng/ µl) genomic DNA as template, 5µl PCR master mix 5X qARTA. Taq Master Mix (1.5mM MgCl₂) from Qartabio (QTMM1.5-200), 1 µl primer (concentration 100pM) and 18 µl of nuclease free water. DNA amplification was performed in a DNA thermal cycler (Biometra, Germany). PCR conditions were set as first cycle of 5 min at 94°C for template denaturation, followed by 40 cycles of 45 sec at 94°C, 45sec at 37°C and 1 min 30 sec at 72°C. An additional cycle of 5 min at 72°C was used for final primer extension. Amplified products were analyzed by electrophoresis on 1.4 per cent agarose gel.

Gel electrophoresis

Agarose powder (SeaKem LE, 50004L) was mixed with 1X TAE buffer (Fermentas, B49) to prepare a solution. 1X TAE buffer was prepared by diluting 50X TAE buffer of Fermentas (Composition: 43.12g Tris, 22g Acetic acid, 2.96g EDTA in 1000ml distilled water). The solution was heated to dissolve agarose. Gel red stain (10000X Biotium-41003) was added (0.3µl in 30ml) to it. The hot, clear agarose solution was poured into the tray and was allowed to cool. After cooling the gel tray was placed in an electrophoresis chamber, which was filled with 1X TAE buffer, covering the gel. This allows electrical current from poles at either end of the gel to flow through the gel. Finally, DNA samples were mixed with 6X loading dye (Fermentas, R0611) in 5:1 ratio and loaded on the gel. Electrophoresis was performed and Gel was observed through UV

trans-illuminator to check for presence of DNA.

Scoring of amplified fragments

The amplified profiles for all the primers were compared with each other and bands of DNA fragment were scored as '1' for presence and '0' for absence, generating '0' and '1' matrix. Per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results and Discussion

Molecular variability of different *Xap* isolates collected from Latur, Osmanabad and Beed district

The detail profile of DNA bands of different primers for three isolates of *X. axonopodis* pv. *Punicae* is presented in Table 3. Total 20 RAPD primers were used to detect the genetic diversity, out of them 5 primers exhibited good amplification with scorable bands. A total of 25 DNA bands were detected by 5 primers, of which, 19 bands were found polymorphic. Among these primers, OPA-09, ABA-05 and ABA-07 were shown 100 per cent polymorphism. Remaining ABA-16 and UBC-478 were shown 60 per cent and 0 per cent polymorphism, respectively. The results of RAPD analysis revealed that a total of 76 per cent polymorphism was found between the isolates, indicating molecular variability among the *X. axonopodis* pv. *punicae* isolates. Information on the banding pattern for all the primers were used to determine genetic distance between the isolates and to construct a dendrogram by using unweighted pair group method (Nei's). Based on the Nei's similarity coefficient a genetic similarity matrix was constructed to

assess the genetic relatedness among the three isolates. The similarity coefficient value was ranged from 0.27 to 0.91 across three isolates indicating high degree of genetic variation.

This ultimately means high range of genetic diversity among the isolates studied. The genetic similarity coefficient to an extent of 1.13 was recorded between *Xap2* and *Xap1* isolates followed by 0.91 between *Xap-3* and *Xap-2* isolates. Least genetic similarity

coefficient 0.27 was observed in between *Xap3* and *Xap1*. The dendrogram constructed by Nei's for RAPD analysis shows that isolates can be grouped into two major clusters viz. A and B (Fig. 3) Cluster A divided into two sub clusters namely cluster A1 and cluster A2. Sub cluster A1 showed isolates, *Xap1* (Yekurga), and whereas Sub cluster A2 having single isolate *Xap-3* (Kej). Cluster B was found distinct from Cluster A containing single isolate *Xap-2* (Killari).

Table.1 DNA concentration of bacterial samples

Sr. no.	Sample code	DNA concentration
01	<i>Xap-1</i>	40.0µg/ml
02	<i>Xap-2</i>	36.4µg/ml
03	<i>Xap-3</i>	53.1 µg/ml
04	<i>Xap-4</i>	50.2µg/ml
05	<i>Xap-5</i>	38.4µg/ml

Table.2 Primers used for PCR amplification of *Xap-1*, *Xap-2* and *Xap-3* samples

Primer	Sequences 5'-3'
OPA09	GGGTAACGCC
ABA05	AGGGGTCTTG
ABA07	GAAACGGGTG
ABA16	AGCCAGGCGAA
UBC 478	CGAGCTGGTC

Table.3 DNA banding profile and per cent polymorphism observed in samples of Latur Osmanabad and Beed district (*Xap-1*, *Xap-2*, *Xap-3*)

Sr. no.	Primer code	Primer Sequences 5'-3'	Total band	Polymorphic band	% Polymorphism
01	OPA09	GGGTAACGCC	6	6	100 %
02	ABA05	AGGGGTCTTG	5	5	100 %
03	ABA07	GAAACGGGTG	5	5	100 %
04	ABA16	AGCCAGGCGAA	5	3	60 %
05	UBC 478	CGAGCTGGTC	4	0	0 %
	Total		25	19	76%

Table.4 Nei's original measures of genetic identity and genetic distance

pop ID	<i>Xap-1</i>	<i>Xap-2</i>	<i>Xap-3</i>
<i>Xap-1</i>	****	0.3200	0.7600
<i>Xap-2</i>	1.1394	****	0.4000
<i>Xap-3</i>	0.2744	0.9163	****

Table.5 Primers used for PCR amplification of *Xap-4* and *Xap-5* samples

Primer	Sequences 5'-3'
OPC03	GGGGGTCTTT
OPC20	ACTTCGCCAC
OPH02	TCGGACGTGA
OPH12	ACGCGCATGT
OPH16	GAGCGTCGAA

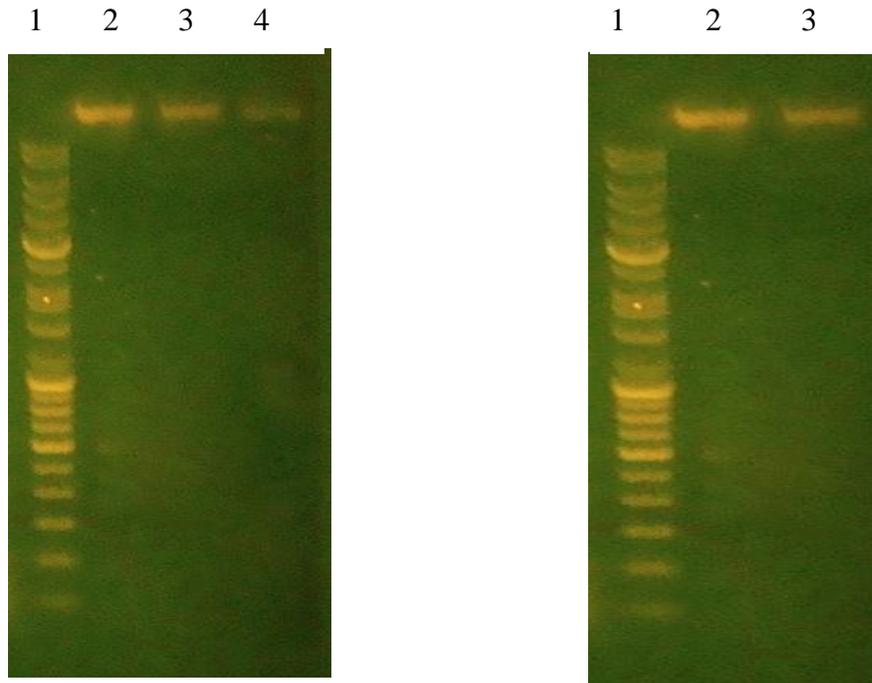
Table.6 DNA banding profile and per cent polymorphism observed in samples of Jalna district (*Xap-4* and *Xap-5*)

Sr. no.	Primer code	Primer Sequences 5'-3'	Total band	Polymorphic band	% Polymorphism
01	OPC03	GGGGGTCTTT	4	4	100 %
02	OPC20	ACTTCGCCAC	11	10	90.91 %
03	OPH02	TCGGACGTGA	7	7	100 %
04	OPH12	ACGCGCATGT	6	5	83.33 %
05	OPH16	GAGCGTCGAA	5	3	60 %
	Total		33	29	87.88 %

Table.7 Nei's original measures of genetic identity and genetic distance

pop ID	<i>Xap-4</i>	<i>Xap-5</i>
<i>Xap-4</i>	****	0.1212
<i>Xap-5</i>	2.1102	****

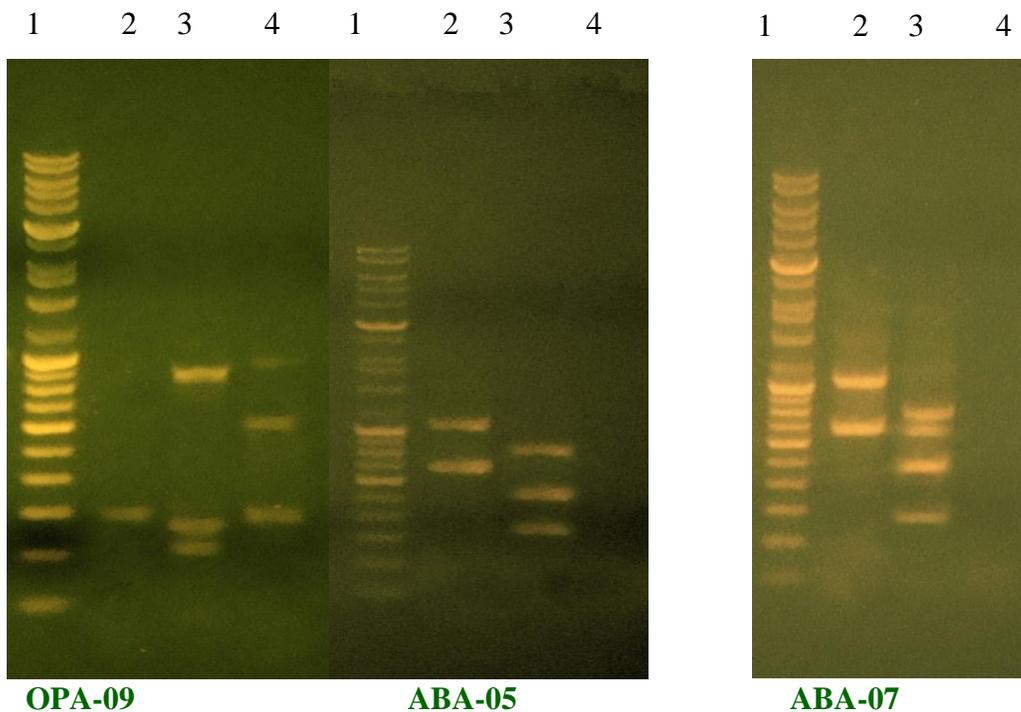
Fig.1 Genomic DNA for the *Xap-1*, *Xap-2*, *Xap-3*, *Xap-4* and *Xap-5* samples



Lane 1: High range DNA marker of GeNei
Lane 2-4: Genomic DNA of *Xap-1*, *Xap-2*, *Xap-3* samples

Lane 1: High range DNA marker of GeNei
Lane 2-3: Genomic DNA for *Xap-4* and *Xap-5* samples.

Fig.2 RAPD profile of *Xap-1*, *Xap-2* and *Xap-3* samples



OPA-09

ABA-05

ABA-07

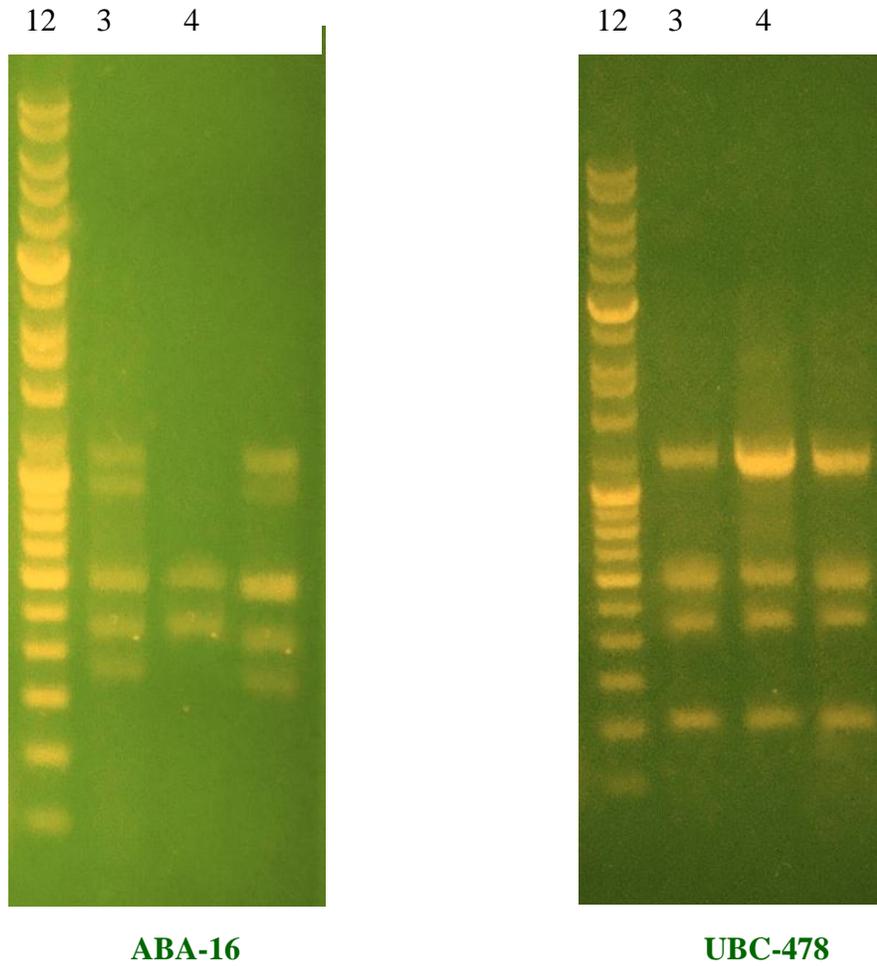


Fig.3 Dendrogram based on Nei's genetic distance

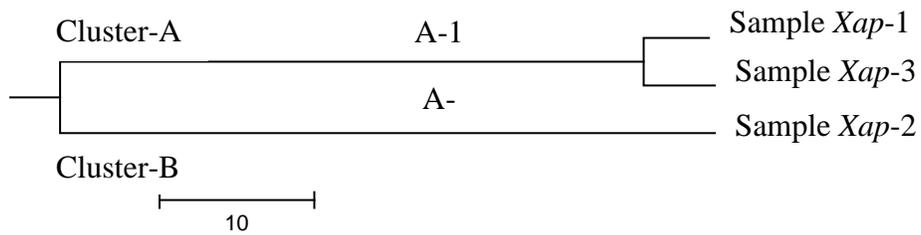


Fig.4 Dendrogram based on Nei's genetic distance

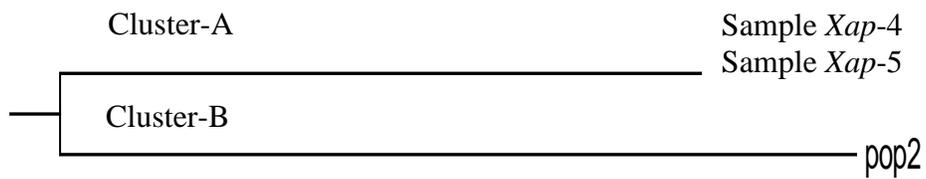
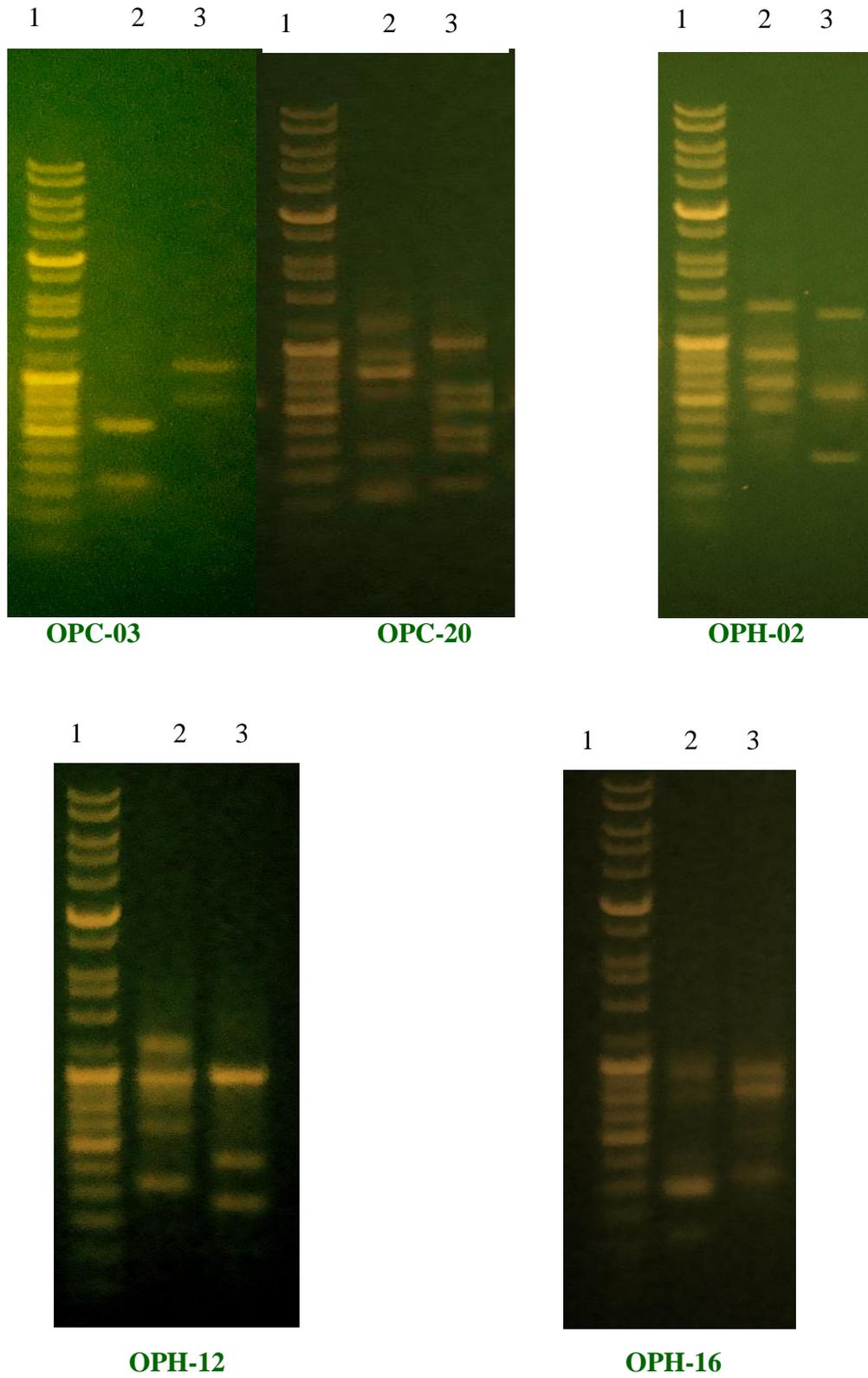


Fig.5 RAPD profile for *Xap-4* and *Xap-5* samples



Molecular variability of *Xap* isolates collected from Jalna district (*Xap-4* and *Xap-5*)

The detail profile of DNA bands of different primers for two isolates of *X. axonopodis* pv. *punicae* is presented in Table 6. The data presented in revealed that total 20 RAPD primers were used to detect the genetic diversity, out of them 5 primers exhibited good amplification with scorable bands. A total of 33 DNA bands were detected by 5 primers, of which, 29 bands were found polymorphic. Among these primers, OPC-03 and OPH-02 were shown 100 % polymorphism. Remaining OPC-20, OPH-12 and OPH1-6 were shown 90.91 %, 83.33 % and 60 % polymorphism respectively. The results of RAPD analysis revealed that a total of 87.88% polymorphism was found between the isolates, indicating molecular variability among the *X. axonopodis* pv. *Punicae* isolates. Information on the banding pattern for all the primers were used to determine genetic distance between the isolates and to construct a dendrogram by using unweighted pair group method (Nei's).

Based on the Nei's similarity coefficient a genetic similarity matrix was constructed to assess the genetic relatedness among the two isolates. The similarity coefficient value ranged from 0.20 to 0.88 across two isolates indicating high degree of genetic variation given in (Table 7). This ultimately means high range of genetic diversity among the isolates studied. The highest genetic similarity coefficient to an extent of 2.11 was recorded between *Xap 4* and *Xap5* isolates followed by 0.12 between *Xap5* and *Xap4* isolates. The dendrogram constructed by Nei's for RAPD analysis shows that isolates can be grouped into two major clusters viz. A and B (Fig.4) Cluster A containing single isolate *Xap 4* (Nimgaon) and another cluster B distinct from cluster B containing single isolate *Xap5* (Badnapur).

These results obtained in present study are in accordance with the reports of many earlier workers. Chakrabarty *et al.*, (2005) standardized a protocol for extraction of genomic DNA from bacteria *Xanthomonas axonopodis* pv. *malvacearum*. Nunes *et al.*, (2009) standardized a simple, quick and easy protocol for extraction of total DNA of the bacteria *Xanthomonas axonopodis* pv. *phaseoli*. William *et al.*, (1990) showed that RAPD reaction mixtures can be created from several arbitrary nucleotide sequences, using short primers when amplified with the PCR. During annealing process, the primer sequences which are not directed to any known genetic locus, attached to the target DNA at random sites through a complementary sequence and permitted for initiation of polymerization. Giri *et al.*, (2011) stated that bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is the devastating disease of pomegranate and the analysis of their RAPD profiles showed a high level of genetic variability among the strains of *X. axonopodis* pv. *punicae*. The cluster analysis based on similarity coefficients separated the sixteen strains into two major clusters. However, result reflected that the variation exhibited by the strains of *X. axonopodis* pv. *punicae* is independent of their geographical location. Gadhe *et al.*, (2016) studied the variability among the five isolates of *Xanthomonas axonopodis* pv. *punicae* by using 10 RAPD primers, out of which 07 primers produces total 26 scorable bands with an average of 3.7 bands per primer. Out of 26 bands, 21 bands were found to be polymorphic and level of polymorphism was 80.76%. The Jaccard's similarity coefficient showed that the isolate Loni (Code Xap I) was found to have higher value of similarity coefficient 0.88 with Astagaon (Code Xap II), whereas Talegaon (Code Xap III) was found to have lower value of similarity coefficient 0.20 with Rahuri (Code Xap V).

On the basis of the present study, it could be concluded that the population of bacterial blight pathogen *X. axonopodis* pv. *punicae* in Jalna, Latur, Osmanabad and Beed district in Maharashtra are genetically heterogeneous and showed high level of genetic variability within a different geographical regions from RAPD profiles.

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