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RESEARCH ARTICLE OPEN ACCESS

Exploitation of the Xanthomonas Repetitive Intergenic Consensus Sequence for the Development of Efficient SCAR Markers for Detection of *Xanthomonas citri* pv. *punicae* in Field Samples of Bacterial Blight-infected Pomegranates

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Abstract

Bacterial blight in pomegranates (*Xanthomonas citri* pv. *punicae* (*Xcp*)), has become a severe concern in recent years. Hence, a molecular analysis of selected *Xcp* isolates and field samples from different locations was conducted. PCR amplification of the *Xanthomonas* Repetitive Intergenic Consensus (XRIC) box yielded 216 bp and 159 bp size bands in all *Xcp* as well as *X. citri* pv. *citri* (*Xcc*) isolates as well as infected field samples. The 16S rRNA gene and XRIC (216 bp) *Xcp* amplicons shared the highest sequence similarity with various *Xcp* and *Xcc* accessions. Two *Xcp*-specific SCAR primers were designed from *in silico* PCR simulation studies. SCAR *Xcp*1-20 and *Xcp*133-152 primers amplified a 152 bp band in all *Xcp* isolates (except one) as well as in infected pomegranate samples, while *Xcc* isolates yielded a 371 bp band. SCAR *Xcp*F/R amplified a 200 bp band in all infected pomegranate samples as well as seven of eight *Xcp* isolates, while the *Xcp* Pune-Daund isolate, along with both *Xcc* isolates, amplified a 350 bp band. KM-*gyr*B primers amplified a 491 bp band in all *Xcp* isolates; and a 375 bp band in both *Xcc* isolates, as well as in 12 of 20 plant samples, with two yielding other-sized bands. *Xac-rpf*-specific primers amplified an expected 581bp band in all *Xcp* and *Xcc* isolates, as well as 14 out of 20 field samples. It can be concluded that the XRIC box amplification and sequencing method enables the rapid identification of various *Xanthomonas* species. Additionally, both SCAR primers developed can be directly used to identify the bacterial blight-infected field samples, facilitating rapid *Xcp* detection.

Keywords: Molecular Diagnosis, Intergenic Hairpin-like Loops, Oily Spot

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is a popular fruit crop grown both for juice and for table purposes. It has proved to be a blessing for dryland farmers of arid/semiarid regions due to its hardiness and versatile adaptability to thrive under low water availability. India contributes to 36 percent of the global production and 30 percent of the global pomegranate trade.[1] Maharashtra State contributes to two-thirds of its pomegranate production.[1]

Among the various diseases infecting pomegranate, bacterial blight caused by *X. citri* pv. *punicae* (*Xcp*) is a serious threat due to its high epidemic potential.[2,3] It was of minor importance until the early 1990s, when it reached epidemic proportions, resulting in yield losses of 60-80 percent.[4] A further outbreak of this disease has been severe and has acquired an epidemic status in the pomegranate belt of peninsular India.[5,6] Later on, it was reported outside of the Indian subcontinent.[7,8]

The disease infects various aerial plant parts including pomegranate fruits which have lower market value and are not suited for export purposes due to strict quarantine regulations. On average, this disease causes 30%-50% losses, with up to 80% losses reported under epiphytotic conditions. Orchard owners are required to destroy the disease-infected plants and orchards. Species of *Xanthomonas* are difficult to eradicate once they infest any area and strict quarantine measures need to be implemented. Therefore, clean cultivation, effective diagnosis and management are recommended.

The traditional identification of *Xanthomonas* includes isolation, purification, and morphological-cultural studies,[9] but some strains of this genus are reportedly difficult to isolate. Classical methods have a few shortcomings, including a lack of sensitivity and specificity. Molecular diagnostic approaches have been used both at the laboratory and field levels. Pathogen-specific primers have been used for the rapid identification of many phytopathogenic bacteria, including *Xanthomonas* with initial reports from the 1990s.[10-12] Plasmid restriction digestion, 16S rRNA gene sequences, intergenic spacer regions, enterobacterial repetitive intergenic consensus (ERIC), repetitive element sequence-based PCR (REP-PCR), box-based PCR, and gene-specific primers have already proved their importance in diversity studies and diagnostic applications in bacterial diagnosis.[13-16] On the molecular evaluation of *Xcp* isolates, their variability was found to be independent of geographic allocation.[17]

XRIC (Xanthomonas Repeat Intergenic Region) are imperfect palindromic repeats, initially identified from genes involved in xanthomonadin pigment biosynthesis.[18] By evaluating sequence information available on the web, bioinformatics tools based on *in silico* PCR simulations can be used efficiently to design primers specific for molecular diagnosis and pathogen detection. However, PCR primers thus designed for diagnosis need to be validated both using pure bacterial cultures and infected plant samples. This study was undertaken for the validation of Sequence Characterized Amplified Regions (SCAR) primers designed from the XRIC sequence, along with a comparison with previously reported related primers in a larger sample size. This method allows for the early detection of phytopathogens such as *Xanthomonas*, in nurseries. The aim of this study was to develop and validate PCR primers specific to *Xcp* as pure isolates as well as bacterial blight-infected samples.

MATERIALS AND METHODS

Collection of pathogen samples

A survey was conducted in the pomegranate-growing regions of western Maharashtra, including Solapur, Nashik, Pune, and Ahilyanagar districts, to collect bacterial blight-infected pomegranate samples (Table 1). Plants were confirmed as infected based on typical symptoms. *Xanthomonas* were isolated from macerated, infected pomegranate samples, streaked on nutrient agar medium with sucrose (NAS) and further incubated at 28 ± 2 °C for 48-72 h.[19] After 48-72 h they were examined for the presence of typical colonies, which were transferred to NAS slants and maintained on yeast extract glucose agar with a charcoal slant for further studies. Eleven pure isolates of *X. citri* pv. *punicae* (*Xcp*) grown on nutrient agar slants were further used for molecular characterization.

Table 1. Bacterial samples used for PCR studies

No.	Species/pathovar	Details of geo-graphical origin
1	*X. citri* pv. *punicae* (*Xcp*)	Ahilyanagar
2	*X. citri* pv.*punicae* (*Xcp*)	Solapur
3	*X. citri* pv. *punicae* (*Xcp*)	Solapur-Akkalkot
4	*X. citri* pv. *punicae* (*Xcp*)	Solapur-Pandharpur
5	*X. citri pv.punicae* (*Xcp*)	Ahilyanagar-Sangamner
6	*X. cirti* pv.*punicae* (*Xcp*)	Nashik-Deola
7	Unpurified culture	Ahilyanagar
8	Unpurified culture	Ahilyanagar
9	Unpurified culture	Ahilyanagar
10	*X. citri* pv *punicae* (*Xcp*)	Ahilyanagar
11	*X. citri pv. punicae* (*Xcp*)	Pune (Daund)
12	*X. citri* pv.*citri* (*Xcc*)	Nagpur
13	*X. citri* pv.*citri* (*Xcc*)	Tamilnadu

These bacterial isolates were maintained on YEM agar slants at 4 °C.

The details of these isolates collected from Western Maharashtra are given in Table 1. A pure isolate from Deola-Nashik was used as a positive control for the confirmation of field samples. Simultaneously, crude extracts of naturally bacterial blight-infected pomegranate samples, as well as artificially inoculated plants, were also used along with two *X. citri* pv. *citri* (*Xcc*) and six isolates of *X. vesicatoria* from different parts of Maharashtra (from another study) for comparative molecular studies. Eleven *Xcp* bacterial isolates were initially evaluated using biochemical tests, *viz*. Gram staining, KOH solubility, Kovac's oxidase, starch hydrolysis, lipase activity, arginine dihydrolase test, gelatin hydrolysis, and catalase tests, as well as for growth on 0.2% asparagines medium. A pathogenicity test was conducted using the standard syringe infiltration method on leaves of one-year-old pomegranate cv. "Ganesh". Freshly grown (48 hrs, 28 °C) bacterial suspension (10^7 to 10^8 CFU/ml) was syringe infiltrated into recently expanded leaves.

Collection of infected pomegranate plant samples for diagnosis

Bacterial blight/oily spot disease-infected pomegranate plant samples were collected separately from the orchards of different pomegranate growing areas of district Nashik (Maharashtra state) i.e., Deola, Malegaon, Satana and Kalwan Tehsils (Table 2). The plant samples taken were freshly infected pomegranate plant leaves showing water-soaked oily coalesced lesions. The collection was done in the early symptoms stage. The infected plant parts were collected from orchards, wrapped in aluminum foil, labeled, and kept in liquid nitrogen till genomic DNA isolation in the laboratory for further studies.

Genomic DNA isolation from *Xanthomonas* pure bacterial cultures

In this investigation, bacterial genomic DNA was isolated from three-loops full of four to five days old culture grown on nutrient agar slants using the Sarkosyl 2% Protease digestion method.[16] It involved dissolving culture pellets in sarkosyl 2% (dissolved in suspension buffer 50 mM Tris, 20 mM EDTA) and 7.5 µl of predigested protease and incubating at 50 °C until the solution had cleared. Phenol-chloroform extraction was followed by isopropanol precipitation of DNA and suspended pellets in 50 µl of TE buffer (10 mM Tris and 1 mM EDTA). In the case of crude extracts, infected tissue debris was removed by brief a spin (10,000 rpm for 3-5 minutes) to avoid blockage of the spin column.

Plant Genomic DNA isolation from infected leaves of pomegranate

In this investigation, twenty bacterial blight-infected pomegranate leaf samples (100 mg each) were homogenized with liquid nitrogen in sterile mortars and pestles, and plant genomic DNA was isolated using HiPurA™ Plant DNA Miniprep Purification kit.

The genomic DNA was quantified on Nanodrop Spectrophotometer (ND-1000) at 260 nm. The genomic DNA band intensity in a 0.8% agarose gel was visualized along with that of standard Lambda phage DNA (50 ng) on a Fluor Chem.™ Alpha Innotech gel documentation unit.

Cloning and sequence analysis of eluted fragments

XRIC (216 bp) and 16S rRNA (1500 bp) DNA fragments from a strain from Deola (District-Nashik) were eluted from the gel, cloned into a PCR cloning vector pTZ57R/T (2886 bp), and transformed into competent *Escherichia coli* strain JM109. Custom Sanger sequencing was done for cloned fragments in a vector. Manual sequence editing was done using the sequence analysis tools

Table 2. Infected plant samples used for PCR studies

Sample No.	Details of bacterial blight infected plant samples studied
#1 to #6	Deola, Nashik District; leaves with water-soaked lesions
#7 to #11	Satana, Nashik District; leaves with water-soaked lesions
#12 to #15	Malegaon, Nashik District; leaves with water-soaked lesions
#16 to #20	Lakhmapur village (Satana, Nashik District), Leaves with water-soaked lesions
#21	Deola, Nashik District
#22	Kalwan, Nashik District
#23	MPKV, Rahuri, Ahilyanagar District

(ChromasLite 2.01 software) and the sequence results were analyzed by BLAST analysis. After sequence annotations, the details were submitted at www.ncbi.nlm.nih.gov as BankIT submissions with GenBank accession numbers viz., FJ827773.1 (for XRIC hairpin loop-forming region genomic sequence) and FJ827774.1 (for 16S-23S ribosomal RNA gene region with ITS spacers).

Computational analysis

Sequenced data from the XRIC region (acc. no. FJ82777) were assessed for all computational analyses. Web-tool Primer3 was used to design the two SCAR primer pairs (XRIC-Xcp_F/R) (F-Primer: 9-28 bp 5'-CAAAACTTACTGCGCAACCA-3' and R-Primer: 187-206 bp 5'-CTAACAAAACGGAGCGAGCA-3') and *Xcp*1-20F + *Xcp*133-152R (*Xcp*1-20F-primer GCGGCTAACAAAACTTACTG and *Xcp*133-152R Primer CCACTGTACGCATCAGATAG).[20] These SCAR primer sets and the XRIC-Box primer, were initially used for *in silico* PCR amplification studies. This *in silico* amplification simulation experiment was carried using the online available prokaryotic genomes sequence data as templates at the site http://insilico.ehu.es/PCR/.[21] For checking amplification possibilities, the genomes of five individual genera from the order Xanthomonadales, as well as twenty other common genera of class Gammaproteobacteria and 40 genome accessions of the genus *Bacillus* (as negative control) were analyzed. The online-generated virtual banding patterns were observed for their uniqueness and applicability in diagnostic applications.

PCR analysis of both bacterial/plant genomic DNA samples

Genomic DNA amplification of both bacterial and plant genomic DNA samples was carried out by PCR amplification in an Eppendorf Master Cycler. The custom synthesized primers used for the PCR amplification in the current investigation are listed in Table 3. Using gradient PCR amplification, the annealing temperature of primer pairs i.e., *Xcp*1-20+*Xcp*133-152, *Xac*01.rpf+*Xac*02.rpf, *Xoo*.TXT+*Xoo*.TXT.4R, *Xcp*-F+Xcp-R, and KM.gyr-5F+KM.gyr-6R was optimized.

The purified genomic DNA extracts of all the genotypes were used as template DNA. The amplification reaction mixture of 20 µl volume comprised of 1-unit *Taq* DNA Polymerase, 1X Buffer B (without $MgCl_2$), 1.2 mM $MgCl_2$, 1.0 mM dNTP, 10 picomoles of each primer, 30 ng template DNA and water. No template DNA was added in a total negative control PCR reaction.

For PCR amplification, the thermal profile comprised of initial denaturation for five minutes at 94 °C, followed by a PCR regime comprising of 40 cycles of denaturation (94 °C), annealing (50-61 °C gradient) and extension (72 °C) for one minute each, followed by final extension for 10 minutes at 72 °C and finally held at 4 °C till samples were retrieval. After standardization of annealing temperature, the amplification was carried out again to get prominent and specific bands, i.e. 16F27 + 16R1525, 16S RNA-specific (61 °C), XRIC (61 °C), *Xcp*1-20 + *Xcp*133-152 (55 °C), *Xcp*F + *Xcp*R (55 °C), KM.gyr5F + KM.gyr-6R (55 °C), *Xac*01rpf. + *Xac*02.rpf (55 °C) and *Xoo*.TXT + *Xoo*.TXT (56 °C), were used with others for the PCR described before.

The PCR products were resolved by 2% agarose gel electrophoresis along with a GeneRuler™ 100 bp DNA ladder. Gel electrophoresis was conducted at 80 volts for one to two hours and bands were visualized and documented in a gel documentation system. PCR profiles were visualized in the gel documentation unit.

RESULTS AND DISCUSSION

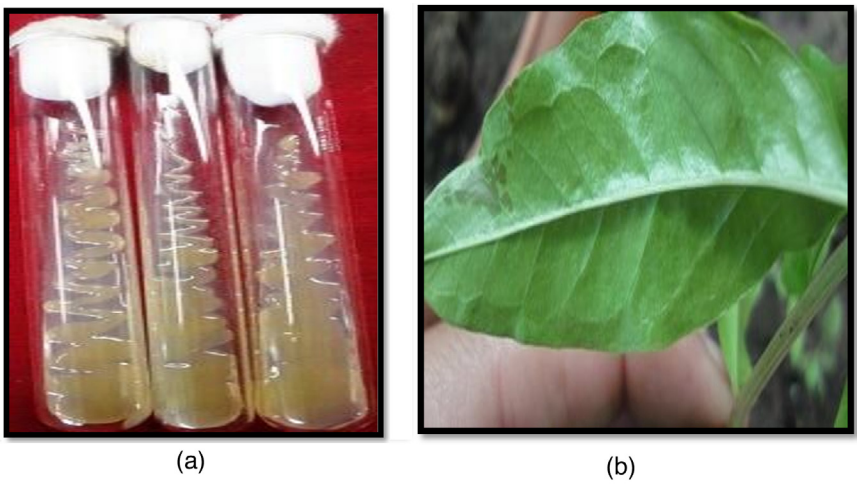
Molecular diagnosis approaches have widescale applications in detecting pathogens alongside conventional diagnosis methods.[22] They can be used for the successful detection of *Xanthomonas* from symptomatic as well as latent infection in host tissues. Detection of symptomless propagation material is of importance because latent infection populations can lead to disease development and severe epidemics under favourable conditions. In the present investigation, a survey was undertaken to detect the pathogen causing bacterial blight (oily spot) in pomegranates by using bacterial pathogen-specific primers.

Microbial and biochemical tests

Microbial phenotype and biochemical characterization of the collected bacterial isolates revealed that the pathogenic bacteria were *Xanthomonas* spp. The collected isolates when grown on NA at 28 °C had smooth, round, yellow-coloured colonies with entire margins (Figure 1a). They were Gram-negative bacteria as they gave pinkish staining with counter stain safranin; which was confirmed by string formation of the culture when using 3% KOH test. All eleven *Xcp* isolates were weakly positive in the oxidase test, positive in the catalase test, hydrolyzed starch as indicated by a clear zone around colonies when iodine stained media was used and showed gelatin liquefaction.

Table 3. PCR primer used for the amplication studies

No.	Primer	Sequence 5' to 3'	Reference
1	16F27	AGAGTTTGATCMTGGCTCAG	Hauben et al.[23]
	16R1525	TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC	16S rRNA specific
2	XRIC	AGAGCGGCTAACAAAACG	Goel et al.[18]
3	*Xcp*-F	CAAAACTTACTGCGCAACCA	SCAR Current investigation
	Xcp-R	CTAACAAAACGGAGCGAGCA	
4	*Xcp*1-20	GCGGCTAACAAAACTTACTG	SCAR Current investigation
	*Xcp*133-152	CCACTGTACGCATCAGATAG	
5	KM.gyr-5 F	GTTGATGCTGTTCACCAGCG	Mondal et al.[24]
	KM.gyr-6R	CATTCATTTCGCCCAAGCCC	
6	*Xac*01.rpf	CGCCATCCCCACCACCACCACGAC	Coletta-Filho et al.[25]
	*Xac*02.rpf	AACCGCTCAATGCCATCCACTTCA	
7	*Xoo*.TXT.F	GTCAAGCCAACTGTGTA	Sakthivel et al.[26]
	Xoo.TXT.4R	CGTTCGCGCCACAGTTG	



(a) (b)

Figure 1. a) Pure culture of *Xcp* on nutrient agar medium used for genomic DNA isolation
b) Bacterial blight infected leaf samples used for DNA isolation

Table 4. Detailed PCR amplification pattern

No.	Primer	Infected plant sample	*Xcp* strain	*Xcc* strain	Exception	Unpurified strain
1	XRIC	159 bp and 216 bp	159 bp and 216 bp	159 bp and 216 bp	-	159 bp+ 216 bp
2	*SCAR Xcp*F+R	~200 bp	~200 bp,	350 bp	350 bp (Pune-Daund)	No amplification
3	*SCAR Xcp*120+ 133-152	152 bp	152 bp,	371 bp,	152 bp, 371 bp, 720 bp (Pune-Daund)	
4	*Xac*01+*Xac*02	581 bp	581 bp	581 bp	-	
5	KM-gyrase	491 bp,	491 bp	375 bp	375 bp, 1088 bp (#8 and 18)	
6	16SF27 + 16SFR1525	1500 bp	1500 bp	1500 bp	-	
7	*Xoo*.TXT + *Xoo*.TXT4R	No amplification	No amplification	No amplification	-	

Similar test results were earlier reported in *Xcp*[16] and *Xcc*.[27] They tested negative for the arginine dihydrolase test: as they turned to the orange-pink colour in the presence of the pH indicator phenol red. No growth was observed on asparagine medium; typical of the presence of *Xanthomonas*; which is a characteristic feature that distinguishes it from closely related pseudomonads.[28]

Pathogenicity test

The *Xcp* isolates were shown to be pathogenic. After artificially inoculating the pomegranate plants, typical water-soaked angular lesions appeared after 7 days. Isolation of the pathogen from artificially infected plants proved Koch's postulate, thereby confirming their pathogenicity. Sharma et al. evaluated various *Xcp* inoculation methods on pomegranates, of which the spray method yielded the most reproducible symptoms.[29] The host specificity of *Xanthomonas* species or pathovars is generally limited.[30]

Molecular characterization

Genomic DNA isolated from thirteen isolates of *Xanthomonas* species [including eleven of *Xcp* from Maharashtra and two *Xcc* isolates (from Tamil Nadu and Nagpur)] as well as twenty infected leaf samples [from four locations of

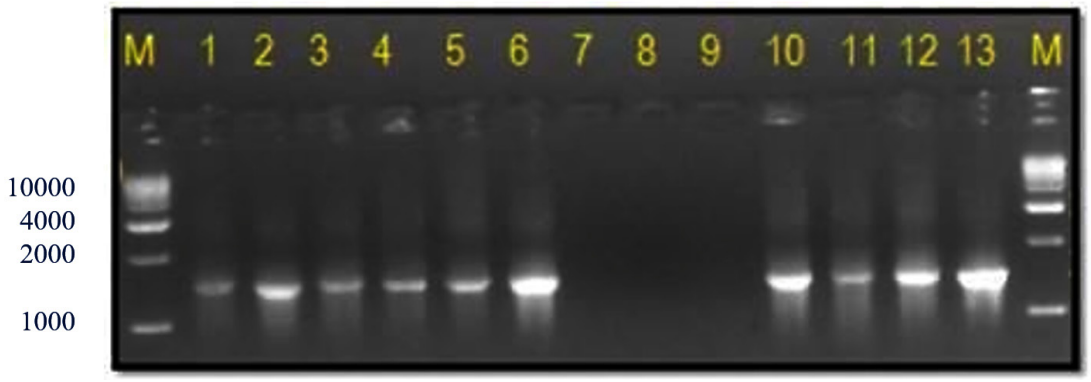


Figure 2. Bacterial genomic DNA amplified using 16S rRNA primers
M: 1 Kb DNA ladder (GeneRuler™); Lanes 1 to 6, 10 and 11: *Xcp* DNA amplified product; Lanes 7 to 9: *Xcp* Unpurified culture DNA amplified products; Lanes 12, 13: *Xcc* DNA amplified products

Nashik district, Maharashtra, India] were diluted to a uniform concentration of 10 ng/µl for use as PCR templates (Figure 1a and b).

In the present study, molecular detection of the bacterial pathogen was undertaken using different primers, specifically SCAR-based markers from the *Xanthomonas* repetitive intergenic consensus (XRIC) sequence as well as those reported previously in literature for *Xcp* and related *Xanthomonas*. Six of the seven primers amplified the products.

16S ribosomal RNA gene-specific PCR amplification studies

The 16S rRNA gene-specific primer pair (16F27 + 16R1525) amplified a 1500 bp fragment in all bacterial samples (except the three unpurified cultures of bacterial samples numbers 7 to 9) (Figure 2) and among 17 of 20 bacterial blight-infected plant DNA samples (except plant samples numbers 2, 4, and 19) (Figure 3) confirming the presence of bacteria.

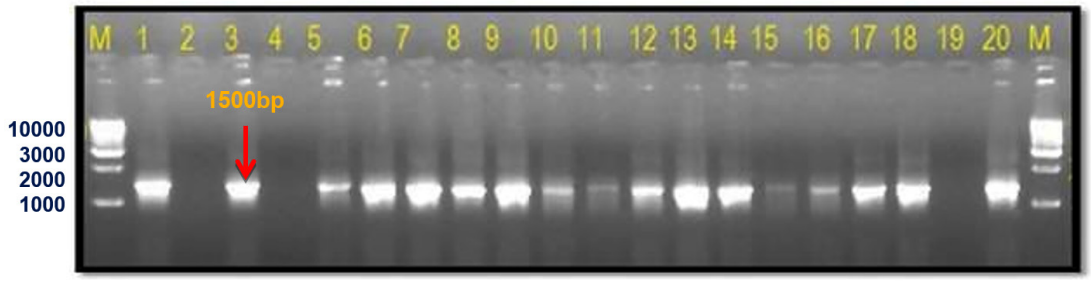


Figure 3. Infected plant DNA amplified using 16S rRNA primers
M: 1 Kb DNA ladder (GeneRuler™); Lane 1 to 20: Infected plant DNA amplified products

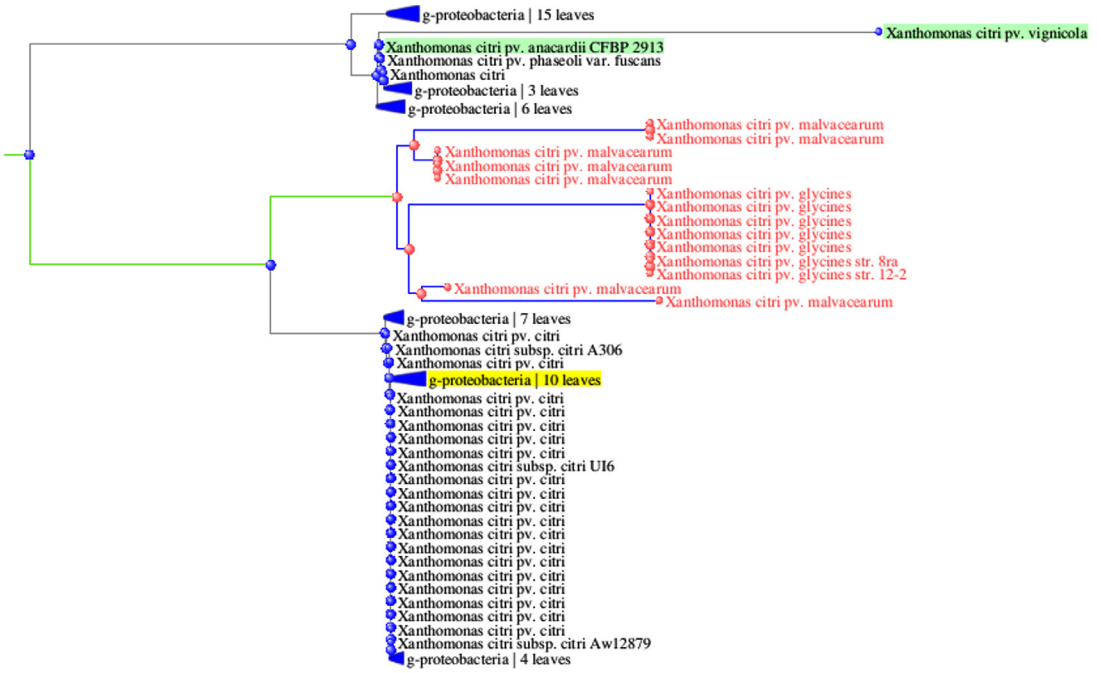


Figure 4. Phylogenetic tree derived 16S rRNA gene sequences of *Xcp* strains

Initially, a 1500 bp 16S rRNA gene band from single isolate *Xcp* Deola-Nashik was cloned and sequenced and a 1018 bp sequence was obtained (GenBank accession FJ827773.1). In a sequence similarity analysis using NCBI megablast (Highly similar sequences), 100 BLAST hits were observed with 98%-99% coverage and 98.53%-99.51% identity. These BLAST results were further subjected to Distance tree results by selecting the Maximum Fast Evolution Method. It revealed that the closest accessions were six *Xcp* strains; which together with 32 *Xcc* accessions formed a subcluster (Figure 4). Another subcluster was comprised of seven *X. citri* pv. *malvacearum* (*Xcm*) and seven *X. citri* pv. *glycine*s (*Xcg*) accessions. This high sequence identity observed in the 16S rRNA gene suggests that *Xcp* and *Xcc* are closely related to *Xcm* and *Xcg*. Another close *X. citri* cluster comprised of accessions of *X.* pv. *phaseoli*; *X.* pv. *vignifola*, and *X.* pv. *perforans*.

16S rRNA gene (1500 bp) sequencing has been extensively used for bacterial characterization and it can assist with bacterial taxonomic resolution.[31-33] Earlier the same primers were used to establish the phylogenetic relationships in the genus *Xanthomonas* which had high sequence similarity (98.2%) of the 16S ribosomal RNA gene.[23] High sequence similarity (95%-98%) was also reported in the 16S-23S rDNA intergenic region between *X. oryzae* pv. *oryzae* and *X. campestris* pathovars.[34]

Extensive molecular studies have reviewed the classification of *Xanthomonas* based on the genomic diversity and relationships therein.[35,36] *X. citri* have been grouped into the subspecies *Xcc* and *Xcm* (both included pathovars

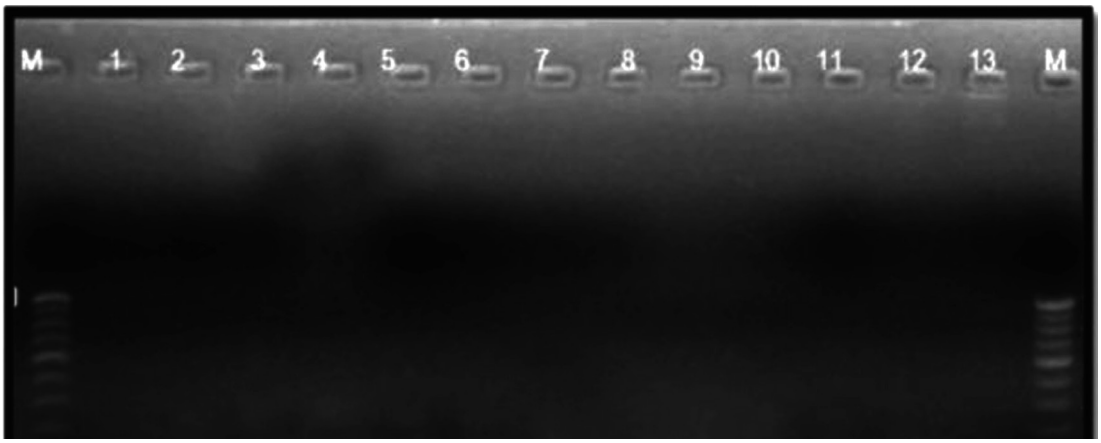


Figure 5. Bacterial genomic DNA amplified using XRIC primer
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 6, 10-11: *Xcp* DNA amplified products; Lanes 7 to 9: Unpurified culture DNA; Lanes 12-13: *Xcc* DNA amplified products

Figure 6. Bacterial blight infected plant DNA amplified using XRIC primer
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 20: Infected plant DNA amplified products

from diverse hosts).[37] It was proposed that *Xcp* is very close to *X. citri* subsp. *malvacearum*.[38] Recently, genome sequences (4.94 Mb) of *Xcp* were found to have 98.78% to >99% nucleotide identity with *X. citri* pv. *citri*.[39,40]

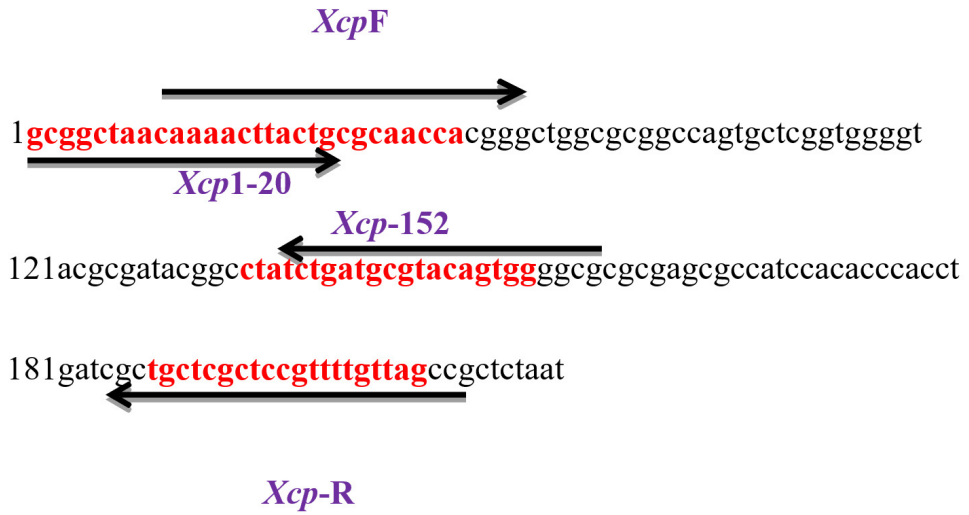


Figure 7. XRIC seq with designed primers

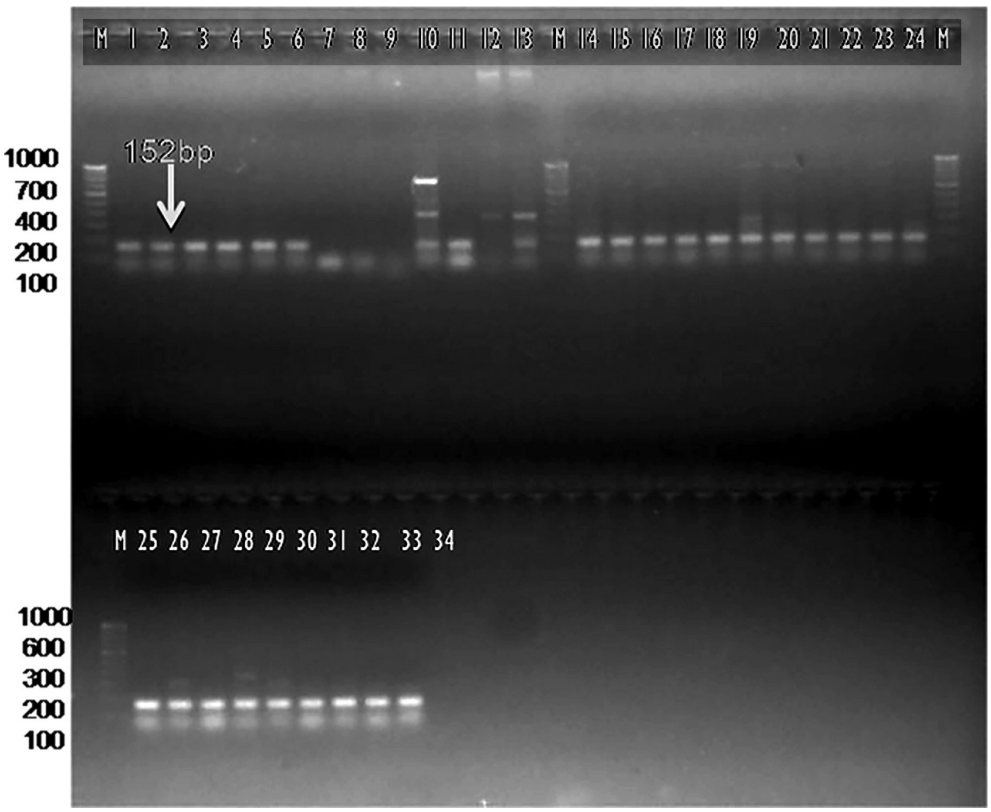


Figure 8. Bacterial and plant DNA amplified using SCAR *Xcp*1-20 + *Xcp*133-152 primer pair
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 6, 10-11: *Xcp* DNA amplified products; Lanes 7 to 9: Unpurified culture DNA; Lanes 12-13: *Xcc* DNA amplified products; Lanes 15 to 34: BB infected plant DNA amplified products

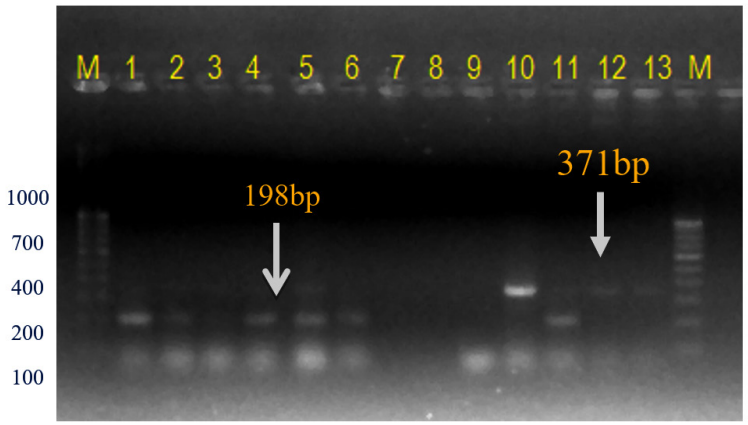


Figure 9. Bacterial genomic DNA amplified using SCAR *Xcp*F + *Xcp*R primer pair
M: Low range 100 bp plus DNA ladder (GeneRuler™); Lanes 1 to 6, 10-11: *Xcp* DNA amplified products; Lanes 7 to 9: Unpurified culture DNA; Lanes 12-13: *Xcc* DNA amplified products

Figure 10. Infected plant DNA amplified using SCAR *Xcp*F + *Xcp*R primer pair
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 20: Infected plant DNA amplified products; Lanes 1 to 20: Infected plant DNA amplified products

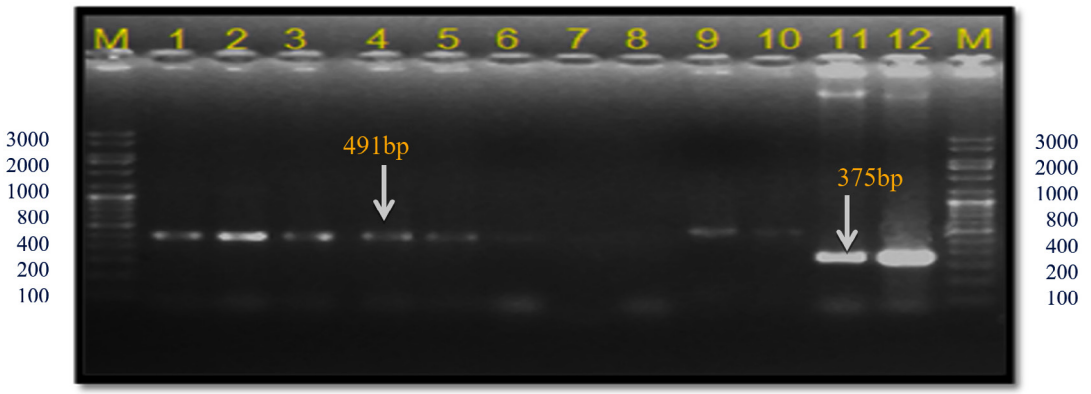


Figure 11. Bacterial genomic DNA amplified using KM.gyr primer pair
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 6, 9-10: *Xcp* DNA amplified products; Lanes 7, 8: Unpurified culture DNA; Lanes 11-12: *Xcc* DNA amplified products

XRIC (*Xanthomonas* Repeat Intergenic Consensus) box amplification and sequence study

Previously, a 145 bp XRIC sequence Box was reported, and was suggested to be *Xanthomonas* specific, and that over a hundred copies of the box may be present in the genome in immediate upstream or downstream regions of various genes.[18] The XRIC box sequence formed a hairpin-like loop structure, with multiple copies including those in the *pigmentation* locus. They further suggested that the 64% G+C content of these XRIC elements was characteristic of *Xanthomonas*. Previously repetitive extragenic palindromic sequence PCR was used for the diagnosis of *X. euvesicatoria*.[15]

During the current investigation, a uniformly monomorphic pattern: with twin fragments of sizes 159 bp and 216 bp were observed in eight *Xcp* pure isolates and two *Xcc* isolates, as well as in twenty infected field samples. The 159 bp and 216 bp product lengths were observed in all three unpurified bacterial cultures (Figure 5). Among the twenty infected plant samples, eighteen of them amplified 159 bp and 216 bp bands, but samples no. 2 and 20 did not amplify (Figure 6). Similar profile was

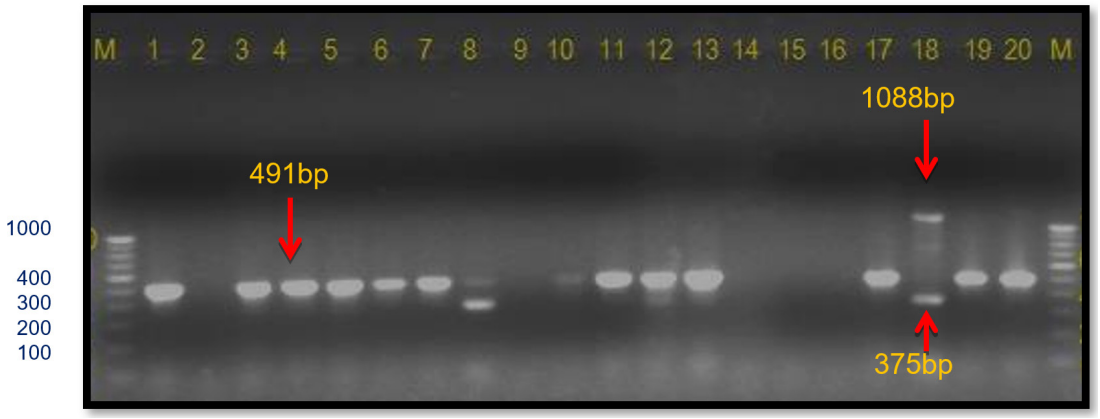


Figure 12. Infected plant DNA amplified using KM.gyr primer pair M: 100 bp DNA ladder; Lanes 1 to 20: Infected plant DNA amplified products

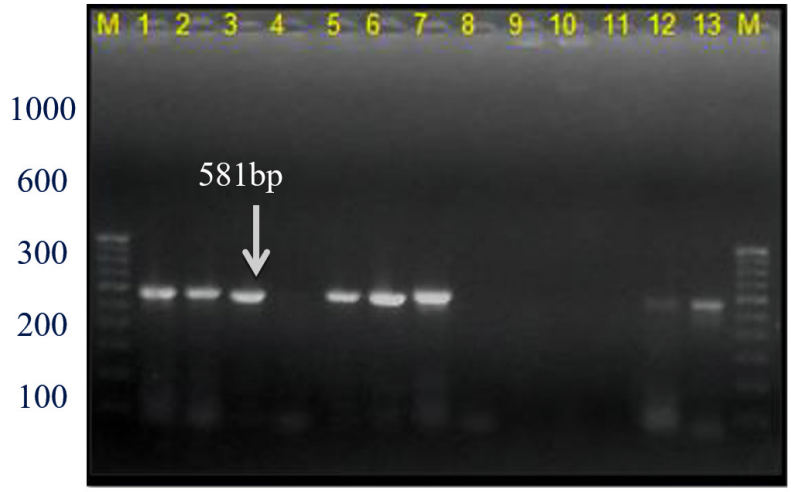


Figure 13. Bacterial genomic DNA amplified using *Xac*01+*Xac*02 primer pair
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 6, 9-10: *Xcp* DNA amplified products; Lanes 7, 8: Unpurified culture DNA; Lanes 11-12: Xcc DNA amplified products

also observed in two crude extracts of naturally infected and the artificially inoculated plant sample (Figure 6). Amplification was not observed in either of the negative controls, i.e., healthy plant DNA or without template DNA. The amplification profile of *X. vesicatoria* isolates varied both in the number of amplicons as well as their polymorphic profile, with their size ranging from 180 bp to 700 bp.

PCR amplification derived 216 bp XRIC band was cloned and sequenced (GenBank accession #FJ827773). In the initial sequencing study, only a single *Xcc* (Strain 603) accession (Hairpin loop forming *Xanthomonas* specific region) showed a significant match. However, during subsequent sequence identity studies using NCBI megablast (Highly similar sequences), fifty-four BLAST hits were observed with 98%-100% coverage and 94.91%-97.18% identity. These 54 similar accessions included 33 sequence accessions from *Xcc*, seven from *Xcp* strains, seven from *Xcg*, six from *Xcm,* and one unclassified *Xanthomonas*. This indicates that the *Xcp*, *Xcc,* and *Xcm* accessions are closely related. In the same analysis (highly similar BLAST), another 32 accessions showed limited sequence similarity with the 3'end region of this accession (for the last 162-216 bp region), with 12%-25% coverage and 85.7%-100% identity; with 18 of those accessions being *X. oryzae* strains and the other 14 accessions being *X. campestris.* The option of somewhat similar sequences (BlastN) yielded a total of 100 BLAST hits with a complete 100% coverage and 76.44%-96.76% identity in various *Xanthomonas*.

Thus, in the present study, the XRIC sequence (accession no. FJ82777) from an *Xcp* isolate revealed that the initial 170 bases were conserved/shared only with closely related *Xcp*, *Xcc, Xcg* and *Xcm*; while in contrast, the last 50 bases of the accession were shared by various other *Xanthomonas*. This suggests that the initial 170 bp region is a good candidate for designing *Xcp* specific primers. As a result, it was proposed that the XRIC based PCR diagnosis can be used in the identification or detection of *Xanthomonas* in field diseased plant samples. Most of the conserved sites were intergenic regions either related to genes encoding pathogenesis-related or hypothetical proteins.

During an *in silico* simulation study of the available database using available prokaryotic genomes, none of the 25-genus other than *Xanthomonas* yielded any amplification, even under less stringent conditions with XRIC primers (results are not presented). These twenty-five-genera included five genera of order Xanthomonadales, i.e., *Stenotrophomonas*; *Xylella*; *Pseudoxanthomonas*; *Frateuria,* and *Dyella*, as well as common pathogenic bacteria like *Pseudomonas*, *Erwinia,* and *Ralstonia*. Under

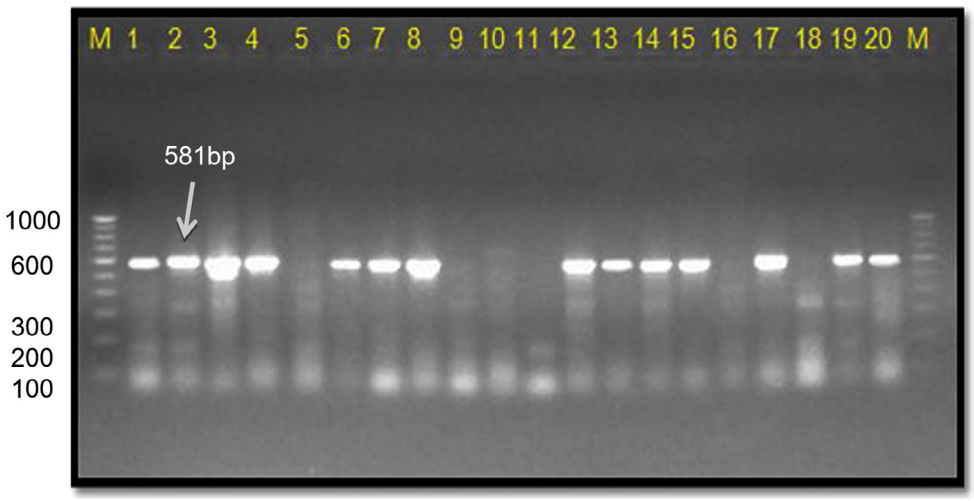


Figure 14. Infected plant DNA amplified using *Xac*01 + *Xac*02 primers
M: 100 bp DNA ladder (GeneRuler™); Lanes 1 to 20: Infected plant DNA amplified products

stringent conditions simulation (no mismatch allowed), XRIC box primer *in silico* amplification was observed in seven species/pathotypes amongst the panel of 14 *Xanthomas*; including three *Xcc* strains (amplified a 144 bp amplicon); three *X. oryzae* pv. *oryzae* (128 bp and 144 bp amplicons) and a *X. oryzae* pv. *oryzicola* (125 bp and 1156 bp amplicons). However, under less stringent *in silico* amplification conditions (two base mismatches allowed with none at the 3' end), all 14 species/pathotypes of *Xanthomonas* yield amplification, with all of them yielding multiple bands, including common amplicons in the range of 142-146 bp.

SCAR based *Xcp* primers for specifically detecting *Xcp*

Based on these findings, that this region is highly pathovar specific, two new pairs of SCAR primers, *Xcp*1-20 + *Xcp*133-152 and *Xcp*F + *Xcp*R, were designed using Primer3 software at the website www.ncbi.nlm.nih.gov (Figure 7). *Xcp*1-20 + *Xcp*133-152 was designed to amplify the 152 bp region by excluding the ~60 bp terminal region shared with different *Xanthomonas* species; while *Xcp*F/R was designed to amplify the almost complete region. Further, *in silico* PCR amplification under stringent conditions using both newly designed SCAR primer sets revealed no amplification in any other member of *Xanthomonas* (including three *Xcc* strains among the panel of 14 members) or *Pseudomonas*. With *Xcp*F + *Xcp*R primers, under less stringent conditions (two mismatches allowed with one in the last 3 nucleotides at the 3' end): an amplicon of size 129 bp was observed in only two members of *Xoo* (KACC10331 and MAFF 311018). The size of those two amplicons observed in this study was significantly smaller than that of 129 bp specific to *Xcp*.

The current investigation was undertaken to further confirm/validate the *in silico* amplification results by further *in vitro* PCR amplification with the designed primer sets. The newly designed SCAR-based primer pair *Xcp*1-20+*Xcp*133-152 amplified a 152 bp band in all *Xcp* isolates and bacterial blight-infected pomegranate samples (Table 4; Figure 8). In no. 10 Pune-Daund *Xcp* isolate, additional 371 bp and 720 bp bands were amplified. In the two *Xcc* isolates (Tamil Nadu and Nagpur isolates) (no. 12 and no. 13) yielded 371 bp bands. Three unpurified cultures, 7, 8 and 9, lacked amplification, likely due to the absence of *Xanthomonas.* All the twenty-disease infected pomegranate leaf samples used for the amplification amplified a 152 bp product (Figure 8).

Another SCAR *Xcp* F/R primer pair amplified a 200 bp band in all blight-infected plant samples, as well as seven of eight *Xcp,* isolates collected from western Maharashtra. Pune-Daund *Xcp* isolate no. 10 amplified a 350 bp band along with both *Xcc* isolates nos. 12-13, while three unpurified bacterial cultures did not amplify (Table 4; Figure 9). All twenty infected pomegranate DNA samples amplified a 200 bp band with primer pair *Xcp*F + *Xcp*R (Figure 10). Under *in silico* conditions, the pathovar-specific primer set, *Xcp*-F/R and *Xcp*1-20 + *Xcp*133-152 didn't amplify in any other *Xanthomonas* except *Xcc.*

Previously seven *Xanthomonas* effector *xop* genes were evaluated for diagnosis, of which *xopQ* primers yielded a 190 bp amplicon in plants infected with *Xcp* and *Xcp*.[41,42] A loop-mediated isothermal amplification (LAMP) technique of *Xcp* was developed and validated in pomegranates using various sets of 16S rRNA primers.[43]

Verification of previously reported *gyrase* (*gyrB*) gene-based primer for detecting *Xcp*

Previously, the *gyr*B gene was used for identifying *Xanthomonas* species.[24] They reported that *Xcp* isolates amplified a 491 bp band. Similarly, a specific 491 bp *gyrB* amplicon was also reported in bacterial blighted (*Xcp*) infected field samples of pomegranate.[6] The identity of the *Xap* isolates was confirmed by specific *gyrB* and 16S rRNA gene sequences, with all isolates resulting in amplicon sizes of the PCR products were 491 bp and 1450 bp respectively.[33] Previously phylogenetic analysis of 203 isolates of *Xanthomonas* was done using their *gyrB* gene sequences.[36]

In the present study, these *gyrB* gene-specific primers KM *gyr*-F/R amplified a 491 bp band size in all *Xcp* isolates; however, in both *Xcc* isolates (no. 11 and 12), a 375 bp band was observed (Table 4; Figure 11). In the unpurified cultures no. 7-9, there was no amplification. The 491 bp was also observed in 12 out of 20 field samples. However, in two samples from the

Satana, different bands were observed, i.e., sample #8 (320 bp) and #18 (491 bp and 1088 bp bands) (Figure 12).

Verification of previously reported *Xac.rpf* primers

Previously, primers were developed for the detection *of Xcc* derived from the *rpf* gene region, in citrus plants.[25] They reported that a 581 bp band was amplified in all *Xcc* infected samples. From the previous sequence similarity studies conducted in our laboratory it was observed that *X. citri* pv. *citri* was the closest pathovar, specifically in terms of sequence identity to *X. citri* pv. *punicae.* The primer set for the ITS of ribosomal RNA gene sequences was highly specific for *X. citri pv. citri*, whereas the *pthA* gene primer set was common to all strains of citrus blight canker *Xanthomonas.*[13] On the contrary, leucine-responsive regulatory protein (*lrp*) gene was informative in distinguishing groups of *Xcc*.[44] It was proposed that citrus canker causing *Xanthomonas* has self-mobilizing plasmids with pathogenicity factors, capable of transferring pathogenicity into other xanthomonad residents on the same citrus plant.[45] A highly reproducible ligation-mediated PCR technique was developed based on three insertion sequences (IS-LM-PCR) in *Xcc.*[46]

This *Xcc-specific Xac.rpf* primer pair amplified a 581 bp product in almost all the *Xcp*/*Xcc* bacterial isolates across various geographical locations, matching the previously reported size (except for three unpurified culture nos. 10, 11, and 12 from the Ahilyanagar region) (Table 4; Figure 13). It also amplified in 14 of 20 bacterial blight-infected pomegranate plant samples, yielding a 581 bp product; while six samples were from Deola and Satana tehsils (no. 6, 10, 11, 12, 17 and 19) did not amplify (Figure 14). These results in the current investigation matched expected sequence similarity studies. However, the *Xac.rpf* primers are not specific to *Xcc* only but they are also amplified in *Xcp* and could not differentiate them from *Xcc* either.[25]

Verification using primer for specifically detecting *X. oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (*Xoo*) based primers developed by earlier workers[26] were included in the current studies as the *in silico* PCR simulation studies with *Xcp* primers had shown amplicons under less stringent conditions (2 mismatches) though of varying sizes in *Xoo*. Insertion sequence IS1112 based PJEL1/2 primers were also used to generate specific and reproducible PCR fingerprint patterns for the *Xoo* isolates.[47] Similarly 16S-23S rDNA spacer-derived primers were designed by researchers that specifically amplified a 470 bp band in all strains of *Xoo*; as well as in other three *Xanthomonas,* including *Xcc*.[34]

In the present study, no amplification was observed with the *Xoo*-specific primer pair *Xoo*.txt+*Xoo*.txt4R in any samples, either from *Xcp* or *Xcc* (Table 4). Similarly, the infected pomegranate DNA samples did not yield any amplification.

CONCLUSION

In this study, we have developed an XRIC box-based PCR assay for the efficient diagnosis of *Xcp* infected samples. The SCAR primers designed from the XRIC box sequence were highly efficient in distinguishing between closely related *Xanthomonas* species. The present investigation showed close sequence similarity as well as cross-amplification between *Xcp* and *Xcc.* This analysis demonstrates the significance of the XRIC sequence in differentiation between *Xanthomonas* pathovars and specific diagnosis of *Xcp*. Currently, this assay is being used for diagnosis and allows for adapting appropriate management measures.

PCR amplification using the XRIC primer yielded two prominent bands of 216 bp and 159 bp in all *Xcp* and *Xcc* isolates as well as plant samples, including crude extracts. XRIC sequence study revealed that the initial 170 bases were highly conserved for *Xcp*, *Xcc, Xcg,* and *Xcm*. Therefore, the initial 170 bp XRIC region was a good candidate region for designing *Xcp*-specific primers. SCAR primer pair (*Xcp*1-20+*Xcp*133-152) thus designed amplified a 152 bp band in all *Xcp* isolates and infected pomegranate samples, except one isolate; while both *Xcc* isolates yielded 371 bp bands. Another SCAR primer pair, *Xcp*F/R amplified a 200 bp and in all bacterial blight-infected plant samples and seven of eight *Xcp* isolates. An *Xcp* isolate along with both *Xcc* isolates amplified a 350 bp band. Reference KM-*gyr*B gene primers amplified a 491 bp and in all *Xcp* isolates, a 375 bp band

in both *Xcc* isolates. However, PCR amplification showed 491 bp in 12 of 20 plant samples. Thus, it could be concluded that primers XRIC, SCAR-based *Xcp*F/R, *Xcp*1-20+*Xcp*133-152, *gyrase* gene-based, and *Xcc*-specific primers will be very useful in the identification or detection of *Xcp* in diseased plant samples of pomegranate from fields. Both SCAR-based primers were designed in the present investigation to identify the bacterial pathogen *Xcp* without isolation of pomegranate bacterial blight culture. Therefore, it can be employed for efficient monitoring of the pathogen. These PCR primers identify the plant pathogen, i.e., bacteria directly from field samples. These results can also help to determine the best diagnostic and taxonomic position of the *Xcp*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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