Documentation of virulence and races of *Xanthomonas citri* pv. *malvacearum* in India and its correlation with genetic diversity revealed by repetitive elements (REP, ERIC, and BOX) and ISSR markers

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Abstract

Thirty-four *Xanthomonas citri* pv. *malvacearum* (*Xcm*) isolates collected from three cotton-growing zones of India were subjected for virulence and race documentation and further correlated with genetic diversity as revealed by repetitive elements [repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements] and intersimple sequence repeat (ISSR)—PCR analyses. Among the 34 isolates tested for virulence on susceptible cultivar LRA 5166, 7 were recorded as highly virulent (HV), 16 were moderately virulent (MV) and 11 were less virulent (LV). Eight different races were recorded by using ten cotton host differentials. Twenty-two isolates (65%) belonged to race 18. Twelve isolates (35%) pertained to races 3, 5, 6, 7, 8, 11 and 13. REP, ERIC, BOX, combined repetitive elements, and ISSR analyses revealed the presence of 7, 10, 9, 11, and 8 clusters, respectively, at similarity coefficient of 0.70 in dendrograms. Principal coordinate analysis (PCoA) exhibited 76.4% and 77.5% cumulative variability for combined repetitive elements and ISSR analyses. ERIC produced the highest polymorphic information content (PIC) value (0.928). A lot of intra-pathovar variability was observed in virulence and genomic fingerprinting among *Xcm* isolates. Many of the isolates grouped based on geographical origin irrespective of virulence or race. The spread of the pathogen races in India might be due to the transport of germplasm lines and seed materials from one place to others.

Keywords Cotton · Genetic diversity · REP-PCR · ERIC-PCR · BOX-PCR · ISSR · Principal coordinate analysis · Repetitive elements · Virulence · *Xanthomonas citri* pv. *malvacearum*

Introduction

Xanthomonas citri pv. *malvacearum* (*Xcm*) is an economically important bacterial pathogen causing angular leaf spot disease/leaf blight disease in cotton (Abdo-Hasan

et al. 2008; Vauterin et al. 2000). The pathogen is currently named as *Xanthomonas citri* pv. *malvacearum* based on DNA analysis of the 16S-23S ribosomal intergenic spacer sequences (Schaad et al. 2007). *X. citri* pv. *malvacearum* colonies appeared as slimy, glistening with pale to deep yellow in colour (Madani et al. 2010). Typical disease symptoms include seedling blight, angular leaf spot, vein blight, black arm, and boll rot based on different plant parts infected with the bacterium (Hillocks 1992). Yield losses have been estimated from 10 to 30% and may exceed 50% in Asia and Africa (Thaxton and El-Zik 2001). Cotton fields infected with bacterial blight have been found to show as much as 80% yield loss in certain areas of the World (Jalloul et al. 2015). In India, bacterial blight of cotton has been recorded in all cotton-growing regions every year with 30% yield loss caused by different *Xcm* races (Patil et al. 2003). Under favourable conditions, yield loss can exceed 50% (Raghavendra et al. 2009). The climatic changes increase the probability of the

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development of virulent strain of pathogen to a particular host (Klanderud et al. 2017). Races of *Xcm* were identified by inoculating them into 10 cotton host differential lines, and so far, 19 *Xcm* races have been described in different parts of the world (Hunter et al. 1968; El-Zik et al. 2001) and this classification was followed in this study. However, Verma and Singh (1975) classified the *Xcm* isolates into 32 races by testing them against 7 cotton differentials. Race 18 is the most virulent and occurs predominantly throughout the world (Thaxton et al. 2001; Verma and Singh 1975). Other races including 20, 21, and 22 were reported from Africa and were classified as highly virulent strains (HVS). HVS of *Xcm* were first reported in Africa (Follin et al. 1988). Meshram et al. (2002) reported 14 different races such as 1–8, 10, 11, 12, 13, 15, and 18 from major cotton-growing regions of India. Gholve et al. (2005) found that among the 27 isolates obtained from the Marathwada region of Maharashtra (India), 89% were race 18 and 11% were race 7.

Apart from traditional pathogenicity assays, morphological and biochemical characterization, the number of genotyping methods has been widely used to study the genetic diversity of the genus *Xanthomonas* (Valverde et al. 2007). A study of the plant–pathogen population structure can provide an insight into genetic diversity, evolutionary history and host adaptation (Vinatzer et al. 2014). Genetic variability study of a bacterial pathogen can aid in its detection and assessment of its taxonomy and epidemiology (Singh et al. 2016). Genomic fingerprinting of repetitive elements has been successfully used to characterize and differentiate closely related strains of bacteria (Versalovic et al. 1991). REP-PCR fingerprinting has been proved to be highly diagnostic and has been used to assess genetic diversity within the bacterial population (Rademaker et al. 2000) including *Xanthomonas* at the pathovar level (Louws et al. 1999). The *Xcm* strains from Brazil were evaluated through repetitive elements (REP, ERIC and BOX) for genetic variability (Oliveira et al. 2011). The genetic diversity of HVS of *Xcm* was evaluated by using rep-PCR (ERIC and BOX) fingerprinting (Zhai et al. 2010). RAPD and ISSR were highly suitable for studying the genetic diversity of *Xcm* isolates (Abdo-Hasan et al. 2008).

The pathogenic variability, race identification, and molecular diversity of *Xcm* isolates have not been described in detail in India. The variability studies will be useful in the development of resistant varieties and effective management strategies. The present study was conducted using 34 *Xcm* isolates collected from three cotton-growing zones of India with following objectives: (1) analyses of pathogenic variability and race distribution of pathogen population; (2) genetic diversity analysis among *Xcm* isolates through repetitive elements and intersimple sequence repeat (ISSR)-PCR;

and (3) correlation assessment between virulence, race and geographical origin of isolates with genomic fingerprinting.

Materials and methods

Sample collection, bacterial isolation, and identification

Cotton leaves with typical symptoms of bacterial blight were collected from three cotton-growing zones of India, comprising North (Punjab state), Central (Maharashtra state) and South (Tamil Nadu, Andhra Pradesh, Telangana and Karnataka states) during the crop seasons of 2015–2016 and 2016–2017 (Fig. 1). The infected leaf tissues were surface sterilized with 1% sodium hypochlorite and washed twice in sterile distilled water. The tissues were allowed to ooze out in sterile water. The bacterial exudates were streaked onto nutrient agar (NA) medium and incubated at 30 °C for 72 h. The isolated single colonies were selected for further study. The bacterium was identified through cultural and morphological characters (Schaad et al. 2001). A total of 34 *Xcm* isolates were obtained from Maharashtra (10 isolates), Tamil Nadu (8 isolates), Punjab (5 isolates), Karnataka (5 isolates), Andhra Pradesh (3 isolates), and Telangana (3 isolates) (Table 1). Isolates were maintained at 4 °C on NA slants for immediate use and stored in 70% glycerol at – 80 °C for future use. The positive control PC-CICR of *Xcm* race 18 was obtained from the Division of Crop Protection, ICAR-Central Institute for Cotton Research, Nagpur, India.

Pathogenicity and virulence of *X. citri* pv. *malvacearum*

Cotton seedlings of the susceptible cultivar LRA 5166 were raised in plastic trays using coco pith as growing medium. The *Xcm* isolates were multiplied in nutrient broth for 24 h. The isolates were inoculated into 20-day-old seedlings at a concentration of 2×10^6 CFU/ml. The spray inoculation method was followed and mild pinprick of leaves was performed before inoculation. Inoculated seedlings were incubated in a plant growth chamber in alternate settings at 28 °C, 90% relative humidity and 3000 lux light intensity for 12 h and 22 °C, 90% relative humidity and without light for 12 h (Labtech—LGC 5101, Daihan Labtech India Pvt. Ltd) in the Department of Plant Pathology. Plants were examined for the appearance of lesions from 3 to 20 day post-inoculation. Per cent disease index (PDI) was calculated according to Sheoraj (1988) using a 0–4 disease rating scale. The isolates were classified as less virulent (LV: 0.00–20.00 PDI), moderately virulent (MV: 20.01–40.0 PDI) and highly virulent (HV: > 40.01 PDI) based on PDI.

Fig. 1 Collection of *X. citri* pv. *malvacearum* from cotton growing zones of India

Biochemical characterization of *X. citri* pv. *malvacearum*

Gram staining was performed to test the gram reaction of isolates. Starch hydrolysis, H₂S production, indole production, KOH solubility and gelatin liquefaction tests were performed according to the procedure given by Schaad et al. (2001).

Race identification using ten host differentials

Ten cotton host differentials such as Acala 44, Stoneville 2B-5/S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4 and DP X P4 (Hunter et al. 1968) were obtained from the Division of Crop Protection, ICAR, Central Institute for Cotton Research, Nagpur, for race determination of 34 *Xcm* isolates. Isolates were inoculated into host differentials raised in pots as per the procedure followed in

Table 1 Location of collection, virulence, and races of *X. citri* pv. *malvacearum*

S. no	State	District	Variety (V)/hybrid (H)	Isolate code	GPS		PDI ^a	Virulence class	Race
					Latitude	Longitude			
1	Maharashtra	Nagpur	Suraj (V)	MNSu	21°02'10"	79°03'22"	66.25	HV	18
2	Maharashtra	Nagpur	Suvin (V)	MNSn	21°02'10"	79°03'22"	44.08	HV	18
3	Maharashtra	Nagpur	DCH 32 (H)	MND	21°02'10"	79°03'22"	53.75	HV	18
4	Maharashtra	Nagpur	RCH 2 BG II (H)	MNR	21°02'10"	79°03'22"	61.50	HV	18
5	Maharashtra	Nagpur	NH 615 (V)	MNN	21°02'10"	79°03'22"	35.17	MV	18
6	Maharashtra	Nagpur	Ankur 3034 BG II (H)	MNA _n	21°02'10"	79°03'22"	33.67	MV	11
7	Maharashtra	Akola	Bunny Bt (H)	MAB	20°42'02"	77°02'13"	19.25	LV	6
8	Maharashtra	Akola	AK 081 (V)	MAA	20°42'02"	77°02'13"	26.58	MV	18
9	Maharashtra	Rahuri	Ajit 199 Bt (H)	MRA	19°20'57"	74°38'09"	34.33	MV	18
10	Maharashtra	Rahuri	NT-Br-02-532 (Culture)	MRN	19°20'57"	74°38'09"	18.58	LV	8
11	Punjab	Bathinda	HS 6 (V)	PBH	30°11'08"	74°56'52"	31.42	MV	18
12	Punjab	Faridkot	NCS 855 BG II (H)	PFN	30°40'31"	74°44'56"	22.33	MV	7
13	Punjab	Faridkot	Ankur 3028 Bt (H)	PFA	30°40'31"	74°44'56"	41.50	HV	18
14	Punjab	Faridkot	Bioseed 6588 BG II (H)	PFB	30°40'31"	74°44'56"	39.42	MV	5
15	Punjab	Faridkot	F-846 (V)	PFF	30°40'31"	74°44'56"	44.50	HV	18
16	Karnataka	Dharward	Suvin (V)	KDS	15°27'36"	75°02'47"	49.33	HV	18
17	Karnataka	Dharward	RHB 1005 (V)	KDR	15°27'36"	75°02'47"	16.42	LV	13
18	Karnataka	Dharward	GSB 41 (V)	KDG	15°27'36"	75°02'47"	28.67	MV	18
19	Karnataka	Dharward	Bunny Bt (H)	KDB	15°27'36"	75°02'47"	22.00	MV	18
20	Karnataka	Dharward	SP-2592 (BG II) (H)	KDS _p	15°27'36"	75°02'47"	36.08	MV	18
21	Tamil Nadu	Coimbatore	Surabhi (V)	TCS _{rb}	11°00'54"	76°55'41"	14.08	LV	3
22	Tamil Nadu	Coimbatore	TCH-1075 (V)	TCT	11°01'12"	76°55'48"	9.17	LV	11
23	Tamil Nadu	Coimbatore	DCH-32 (V)	TCD	11°01'12"	76°55'48"	21.58	MV	18
24	Tamil Nadu	Coimbatore	Suraj (V)	TCS	11°00'54"	76°55'41"	26.67	MV	18
25	Tamil Nadu	Coimbatore	BGDS-1063 (V)	TCB	11°01'09"	76°55'49"	18.67	LV	13
26	Tamil Nadu	Coimbatore	LRK 516 (V)	TCL	11°00' 54"	76°55'41"	27.67	MV	18
27	Tamil Nadu	Dindigul	RCH 659 BG II (H)	TDR	10°23'23"	77°49'33"	7.67	LV	8
28	Tamil Nadu	Dindigul	Jackpot (Bioseed) (H)	TDJ	10°25'06"	77°48'46"	11.42	LV	3
29	Andhra Pradesh	Kurnool (Nandhyal)	RCH 659 BG II (H)	ANR	15°27'05"	78°36'17"	22.50	MV	18
30	Andhra Pradesh	Guntur	Darya Bt (H)	AGD	16°24'08"	80°25'54"	14.42	LV	11
31	Andhra Pradesh	Guntur	RCH 659 BG II (H)	AGR	16°28'11"	80°20'02"	32.92	MV	18
32	Telangana	Warangal	RCH 659 BG II (H)	TWR	18°02'49"	79°38'34"	23.42	MV	18
33	Telangana	Warangal	Bindass BG II (Bioseed) (H)	TWB	18°03'55"	79°41'32"	17.58	LV	13
34	Telangana	Hyderabad	Variety	THV	17°32'19"	78°41'96"	12.83	LV	18
35	Maharashtra	Nagpur	Suraj (V)	PC-CICR	21°02'10"	79°03'22"	54.67	HV	18

^aPDI: 0.00–20.00—less virulent (LV); 20.01–40.00—moderately virulent; > 40.01—highly virulent

pathogenicity test. Three replications were maintained for each isolate. Fifteen day post-inoculation, the different races of isolates were identified based on the presence or absence of symptoms on specific host differentials.

Molecular confirmation of *X. citri* pv. *malvacearum* through housekeeping genes

Genomic DNA was isolated from 34 *Xcm* isolates as proposed by Chen and Kuo (1993). *Xanthomonas*-specific six

housekeeping gene primers such as ATP synthase subunit beta (*atpD*), molecular chaperone protein (*dnaK*), elongation factor (*efp*), glutamine synthetase type 1 (*glna*), gyrase subunit B (*gyrB*), and Ton B-dependent receptor (*fyuA*) have been used for the confirmation of *Xcm* isolates at the genus level through PCR analysis (Mhedbi-Hajri et al. 2013). Furthermore, the *Xcm* isolates were confirmed at the pathovar level through pathogenicity test. Among the *Xcm* races, race 18 is most virulent, economically important and all the resistance breeding programmes

were directed against race 18 of *Xcm* all over the world except Africa. Five representative race 18 isolates (MNSu, MND, MNR, TCD, and TCL) were carried forward for sequencing of housekeeping genes and confirmation at the pathovar level. Sequences were submitted at NCBI.

Genetic diversity of *X. citri* pv. *malvacearum*

REP, ERIC, and BOX-PCR (repetitive elements) analyses

Repetitive extragenic palindromic (Rep), enterobacterial repetitive intergenic consensus (ERIC), and BOX

sequence-specific primers (Versalovic et al. 1994) were used for the genetic diversity analyses of *Xcm* isolates (Table 2). PCR amplification of the REP region was performed with the primer pair REP1R-I and REP2-I and the BOX region was performed with the primer BOXA1R by following the cycling conditions (Table 2) mentioned by Dombek et al. (2000). PCR amplification of the ERIC region was carried out with the primer pair ERIC1R and ERIC2 as per the cycling conditions given by Asgarani et al. (2015) with slight modifications (Table 2).

Table 2 Repetitive elements (REP, ERIC, and BOX), ISSR primers and PCR conditions used

Primers	Sequence	References	PCR conditions	References
Repetitive elements				
REP	REP1R-I-5'- IIICGICGICATCIGC-3'	Versalovic et al. (1994)	Initial denaturation—95 °C for 2 min	Dombek et al. (2000)
	REP2-I-5'-ICGICTTATC IGGCCT AC-3'		Denaturation – 94 °C for 30 s Annealing – 40 °C for 1 min Extension – 65 °C for 8 min Final extension—65 °C for 8 min	
ERIC	ERIC1R-5'-ATGTAAGCT CCTGG GGATTCAC-3'	Versalovic et al. (1994)	Initial denaturation—94 °C for 3 min	Asgarani et al. (2015)
	ERIC2-5'-AAGTAAGTG ACTGGG GTGAGCG-3'		Denaturation – 94 °C for 30 s Annealing – 55 °C for 30 s Extension – 72 °C for 30 s Final extension—72 °C for 10 min	
BOX	BOXA1R-5'-CTACGG CAAGG C GACGCT GA CG-3'	Versalovic et al. (1994)	Initial Denaturation- 95 °C for 2 min Denaturation – 94 °C for 30 s Annealing – 52 °C for 1 min Extension – 65 °C for 8 s Final extension- 65 °C for 8 min	Dombek et al. (2000)
ISSR primers				
(GACAC) ₃	5'-GACACGACACGA CAC-3'	Archana et al. (2017)	Initial Denaturation- 94 °C for 2 min Denaturation – 94 °C for 10 s Annealing – 50 °C for 10 s Extension – 72 °C for 10 s Final extension- 72 °C for 10 min	Abdo-Hasan et al. (2008)
(AGG) ₅	5'-AGGAGGAGGAGG AGG-3'	Archana et al. (2017)		
D2	5'-CAGCAGCAGCAG CAG-3'	Abdo-Hasan et al. (2008)		
B1	5'-CTCTCTCTCTCTCTC TTG-3'	Abdo-Hasan et al. (2008)		
B16	5'-GACAGACAGACA GACA-3'	Abdo-Hasan et al. (2008)		
B7	5'-GTGGTGGTGGC-3'	Abdo-Hasan et al. (2008)		
A31	5'-AGCAGCAGCAGCR-3'	Abdo-Hasan et al. (2008)		
A16	5'-CACACACACACAR-3'	Abdo-Hasan et al. (2008)		
B13	5'-CAACAACAACAA CAA-3'	Abdo-Hasan et al. (2008)		

Intersimple sequence repeat analysis

The ISSR analysis was performed with nine different primers (Table 2) for analysing genetic variability among *Xcm* isolates. Samples were subjected to PCR amplification with the cycling conditions (Table 2) mentioned by Abdo-Hasan et al. (2008).

PCR amplifications were carried out in 10 µl reaction volume containing 5 µl of PCR master mix (Smart prime 2X master mix-Red), 2 µl of rep or ISSR primer (Sigma-Aldrich Inc., USA), 2 µl of sample DNA, and 1 µl of sterile water. The PCR products were separated in 1.5% agarose horizontal gel electrophoresis using 80 V power supply for 2 h in 1X Tris–Borate–EDTA (TBE) buffer. The bands were visualized on a UV transilluminator and documented using gel documentation system (Alpha Innotech Corporation, California). Ethidium bromide was used for staining of PCR products. Upon gel electrophoresis, significant differences in the distribution of genetic profiles were determined using NTSYS-PC (2.02i) software (Rohlf 1997). Dendrograms were calculated with Jaccard's similarity coefficient with unweighted pair group method clustering (UPGMA) for independent and combined REP, ERIC, and BOX amplifications and ISSR analysis.

Polymorphic information content and principal coordinate analysis

The polymorphic information content (PIC) value was calculated using the formula developed by Anderson et al. (1993):

$$PIC_i = 1 - \sum P_{ij}^2$$

where P_{ij} is the frequency of the j th allele for the locus i and is summed across all the alleles.

Principal coordinate analysis (PCoA) was performed using NTSYS-PC software 2.02i (Rohlf 1997) through extraction of eigenvalues and eigenvectors. The dissimilarity among the isolates was presented in two dimensions.

Results

Isolation and identification

From the 3 cotton-growing zones of India, 34 *Xcm* isolates were isolated from various cotton cultivars expressing typical bacterial blight symptoms (Table 1). The colonies appeared on NA medium 72 h after incubation. The *Xcm* isolates produced pale yellow, round, convex, mucoid colonies with a glistening texture. All the isolates showed Gram negative reaction. They also recorded positive for Starch

hydrolysis, H_2S production, KOH solubility and Gelatin liquefaction and negative for Indole production tests.

Pathogenicity, virulence, and race identification

All the isolates were pathogenic to the susceptible cultivar LRA 5166. Water-soaked lesions on the lower surface of the leaves appeared 7 days after inoculation. Angular leaf spots, vein blight, and black arm symptoms were produced in advanced stages of disease progression. PDI varied from 7.67 to 66.25 among the 34 isolates studied (Table 1).

The 34 isolates were categorized into 7 HV isolates, 16 MV isolates, and 11 LV isolates (Table 1). The MNSu isolate was recorded with the highest PDI of 66.25 followed by the MNR isolate which recorded with 61.50 PDI. Both the isolates were isolated from Maharashtra and belonged to the HV category. The lowest PDI of 7.67 was recorded in TDR isolate from Tamil Nadu and belonged to the LV category. Among 34 *Xcm* isolates screened, 8 different races such as 3, 5, 6, 7, 8, 11, 13 and 18 were recorded and a maximum of 22 isolates belonged to race 18 (64.7%) (Table 1; Fig. S1).

Molecular confirmation of *X. citri* pv. *malvacearum*

Xanthomonas-specific housekeeping genes including *atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, and *fyuA* in 5 representative isolates belonging to race 18 were sequenced and characterized. All the housekeeping genes of the isolates had 100% similarity with the respective gene sequences available in the NCBI database. The sequences were submitted to NCBI and accession numbers were obtained (MNSu—*atpD*: KY851295, *dnaK*: MF152719, *efp*: MF152724, *glnA*: MF171135, *gyrB*: MF171140 and *fyuA*: MF171145; MND—*atpD*: KY865094, *dnaK*: MF152720, *efp*: MF152725, *glnA*: MF171136, *gyrB*: MF171141 and *fyuA*: MF171146; MNR—*atpD*: KY865095, *dnaK*: MF152721, *efp*: MF152726, *glnA*: MF171137, *gyrB*: MF171142 and *fyuA*: MF171147; TCD—*atpD*: KY865096, *dnaK*: MF152722, *efp*: MF152727, *glnA*: MF171138, *gyrB*: MF171143 and *fyuA*: MF171148; TCL—*atpD*: MF152718, *dnaK*: MF152723, *efp*: MF152728, *glnA*: MF171139, *gyrB*: MF171144 and *fyuA*: MF171149).

Genetic diversity of *X. citri* pv. *malvacearum*

REP, ERIC, and BOX-PCR (repetitive elements) analyses

REP, ERIC, and BOX-PCR (repetitive elements) analyses collectively yielded 428 bands for 34 *Xcm* isolates and positive control (PC-CICR). Independent REP, ERIC, and BOX-PCR yielded 130, 171, and 127 bands, respectively. Dendrograms were analysed at the 0.70 similarity coefficient for cluster formation among isolates.

Table 3 Allele number, polymorphism content, and PIC values for repetitive elements (REP, ERIC, and BOX-PCR) and ISSR primers in *X. citri* pv. *malvacearum*

Primers	No. of alleles	No. of polymorphic alleles	Per cent polymorphism	PIC value
Repetitive elements				
REP	16	16	100	0.909
ERIC	20	20	100	0.928
BOX	11	11	100	0.885
Combined REP, ERIC and BOX	47	47	100	0.970
ISSR primers				
(GACAC) ₃	13	13	100	0.879
(AGG) ₅	9	9	100	0.835
D2	8	8	100	0.847
B1	8	8	100	0.798
B16	8	8	100	0.811
B7	7	7	100	0.825
A31	7	7	100	0.805
A16	5	5	100	0.744
B13	4	4	100	0.689

PIC polymorphic information content

Rep-PCR-amplified banding patterns were clearly distinguishable with a size varying from 100 to ~5000 bp (Fig. S2). Sixteen different alleles were scored for the 34 *Xcm* isolates. Abundant diversity was observed among the isolates with a PIC value of 0.909 (Table 3). Seven clusters formed at the 0.70 similarity coefficient (Fig. S3). Isolates from south zone belonged either to the MV or to the LV category. The major cluster VII included 17 (50%) isolates. Among them, eight (80%) isolates were from Maharashtra, four (50%) isolates were from Tamil Nadu, three (60%) isolates were from Karnataka and one each from Punjab and Andhra Pradesh. The ~5000 bp unique single band was observed for the THV isolate (race 18 and MV category). The specific band of 1500 bp was obtained for the three isolates MNSn, MRA and TWB. The monomorphic band of 300 bp for 22 isolates and 400 bp for 16 isolates was also recorded (Fig. S2).

ERIC-PCR produced bands from 100 to 3000 bp with 20 different alleles (Fig. S4). The PIC value was 0.928 (Table 3). Ten clusters formed at the 0.70 similarity coefficient (Fig. S5). The major cluster X had 15 isolates. Seven (70%) isolates from Maharashtra including PC-CICR, four (50%) from Tamil Nadu, two (40%) from Karnataka, and one each from Andhra Pradesh and Punjab were recorded. Other isolates were distributed in nine different clusters. A unique single band of 3000 bp was obtained for the PFF isolate and a band of 2000 bp for MNAn, MRN, and TCSrb isolates.

Monomorphic bands of 400 and 600 bp were observed for 22 and 19 isolates, respectively (Fig. S4).

BOX-PCR analysis of 34 *Xcm* isolates revealed the polymorphic banding pattern ranging from 100 to 1200 bp with 11 different alleles (Fig. S6). The PIC value was 0.885 (Table 3). Nine clusters were obtained at the 0.70 similarity coefficient (Fig. S7). The major cluster V recorded with 15 isolates. Most of the north and south zone isolates clustered in this group except the central zone isolates. Eight isolates (80%) from Maharashtra and one from Tamil Nadu (TCL) were grouped in cluster IX. The 1000 bp unique single band was recorded for isolates MRA and THV. The monomorphic band of 500 bp was recorded for 20 isolates and 350 bp another set of 20 isolates (Fig. S6).

Combined REP, ERIC, and BOX-PCR analyses

Comparative analysis of repetitive elements (REP, ERIC, and BOX) revealed that the maximum polymorphic alleles (20) were generated in ERIC followed by REP (16) and BOX (11), suggesting that ERIC was more informative in assessing the genetic diversity of *Xcm* isolates. The PIC value for the combined REP elements was 0.970 (Table 3). Eleven clusters formed at the 0.70 similarity coefficient (Fig. 2). Cluster VI comprised of four isolates (MRN, PBH, TCB, and KDS) from all three cotton-growing zones with HV, MV, and LV categories. The positive control (PC-CICR) shared a homology of 79% with the ANR (race 18 and MV category) isolate from Andhra Pradesh. Thirteen isolates were grouped in the major cluster XI. It included eight (80%) isolates from Maharashtra, three (TCS, TCT, and TDR) from Tamil Nadu and two (KDG and KDB) from Karnataka.

Intersimple sequence repeat analysis

The ISSR primers generated a banding pattern ranging from 100 to 1000 bp. Nine primers produced 69 rows with a total of 549 bands. The PIC value for nine ISSR primers varied from 0.879 to 0.689 with an average of 0.804 (Table 3). The highest PIC value of 0.879 was recorded for primer (GACAC)₃ followed by 0.847 for primer D-2. Among the ISSR primers, (GACAC)₃ generated as many as 13 polymorphic alleles followed by the primer (AGG)₅ with nine alleles. Primers B-1, B-16, and D-2 generated eight polymorphic alleles. Primer (GACAC)₃ was found to be the best for studying genetic diversity among *Xcm* isolates. Eight clusters formed at the 0.70 similarity coefficient (Fig. 3). Twenty-six isolates (74%) were clustered in two major clusters (IV and VIII). Fifteen isolates were grouped in cluster IV, which includes isolates from Punjab, Karnataka, Tamil Nadu, Telangana and Andhra Pradesh. None of the isolates from Maharashtra were placed in this cluster except the positive control. Nine isolates (90%) from Maharashtra and two

Fig. 2 Dendrogram based on cluster analysis (UPGMA) of the estimate of genetic similarity by combined REP, ERIC, and BOX-PCR of *Xcm* isolates. Clusters are shown at 0.70 similarity coefficient. (Virulence class: *HV* highly virulent, *MV* moderately virulent, *LV* less virulent)

Fig. 3 Dendrogram based on cluster analysis (UPGMA) of the estimate of genetic similarity by ISSR-PCR of *Xcm* isolates. Clusters are shown at 0.70 similarity coefficient. (Virulence class: *HV* highly virulent, *MV* moderately virulent, *LV* less virulent)

Table 4 Total variability distributions on different principal coordinates for *X. citri* pv. *malvacearum* in combined REP-PCR and ISSR

Principal coordinates	Eigenvalue	Percentage variability	Cumulative variability
Combined rep-PCR			
1	29.7	63.2	63.2
2	2.9	6.2	69.5
3	1.8	3.8	73.4
4	1.4	3.0	76.4
ISSR-PCR			
1	45.9	66.5	66.5
2	3.2	4.7	71.2
3	2.5	3.7	74.9
4	1.7	2.5	77.5

isolates (PFB and PBH) from Punjab were grouped in the major cluster VIII. Primer B-1 generated identical bands among the isolates based on the location of collection. The primers (GACAC)₃ and (AGG)₅ were found to be better in producing more number of polymorphic bands. Other primers such as A-16, A-31, B-7, and B-13 generated less number of polymorphic alleles among *Xcm* isolates.

Principal coordinate analysis

The PCoA was performed with combined REP, ERIC, and BOX-PCR. The PCoA revealed high per cent variability (63.2%) with the eigenvalue of 29.7. Coordinates 1, 2, 3, and 4 exhibited 76.4% cumulative variability with > 1 eigenvalue for all coordinates (Table 4; Fig. 4a). Likewise, PCoA analysis with ISSR primers was performed. Principal coordinate 1 yielded 66.5% variability with the highest eigenvalue of 45.9. Principal coordinates 1–4 exhibited 77.5% cumulative variability with an eigenvalue of > 1 for all coordinates (Table 4; Fig. 4b).

Discussion

Cotton bacterial blight is a well-established disease and causes severe yield losses in the entire cotton-growing zones of India. However, knowledge of the genetic diversity of Indian *Xcm* isolates is lacking. Research into genomic fingerprinting of 34 *Xcm* isolates in India with repetitive elements (REP, ERIC, and BOX) and ISSR-PCR analyses revealed polymorphism in the genetic make-up of isolates. Rep-PCR was suitable for high-throughput analysis owing to its speed, simplicity and accuracy (Dombek et al. 2000). Adoption of *Xanthomonas* isolates to a specialized niche revealed unique distribution of repetitive elements in their genome in finger printing analyses

(Louws et al. 1994). Analysis of 34 *Xcm* isolates adapted to different agroclimatic zones of India revealed the existence of 7, 10, 9, 11, and 8 clusters for REP, ERIC, BOX, combined repetitive elements and ISSR-PCR analyses, respectively. Genomic fingerprinting of 28 *X. campestris* pv. *campestris* of cole crops collected from Northern India using the rep-PCR technique grouped the isolates into eight clusters (Singh et al. 2015). Clustering through the combination of REP, ERIC, and BOX-PCR profiles reflected the genetic relation of *Xcm* isolates more precisely (Zhai et al. 2010). Similarly, in the present investigation, combined analysis of the repetitive elements (REP, ERIC, and BOX) revealed 11 different clusters among 34 *Xcm* isolates.

In comparison, ERIC-PCR exhibited a high degree of polymorphism among isolates than REP and BOX-PCR. These results are in agreement with the findings of Lopez et al. (2006) in *X. axonopodis* pv. *phaseoli* and Jensen et al. (2010) with *X. campestris* pv. *campestris*. Combined analyses of REP, ERIC, and BOX-PCR produced more similar banding patterns among field strains of *X. campestris* pv. *viticola* from Brazil and ERIC showed the highest polymorphism than the other two analyses (Trindade et al. 2005). ERIC-PCR generated as many as 20 reproducible bands followed by REP (16) and BOX (11). Repetitive sequences (REP, ERIC, and BOX) were placed in different loci of the bacterial genome and each region of amplification provided an independent molecular fingerprinting pattern (Louws et al. 1999). ERIC-PCR analysis of 37 *Xcm* strains from Iran had 21 reproducible bands from 250 to 2500 bp (Madani et al. 2010).

The common banding pattern of amplified bands revealed a close genetic relationship between the isolates. Eighty per cent of the isolates from Maharashtra were grouped together in REP, BOX, and combined repetitive element analyses. In ISSR analysis, 100% of isolates from Maharashtra clustered together. Other isolates from north and south zones were distributed in all other clusters and 30–40% of the isolates from the same geographical origin clustered together irrespective of race or virulence. Geographical origin played a crucial role in the virulence and genetic make-up of the isolates. Conducive weather conditions in the Nagpur region of Maharashtra recorded the maximum number of highly virulent (HV) isolates. Less virulent *Xcm* isolates were often isolated from the United States, and HVS from Africa were evidence for correlation of geoclimatic origin of the strain with virulence (Zhai et al. 2010). These results emphasized the relationship of geoclimatic origin with virulence of isolates. However, Singh et al. (2016) did not observe any correlation between fingerprints and races, hosts or geographical origin among 217 *X. campestris* pv. *campestris* isolates from 12 different crops in India analysed through repetitive elements.

Fig. 4 **a** Principal coordinate analysis (PCoA) for combined REP, ERIC, and BOX-PCR. Coordinates 1, 2, and 3 revealed 73.4% variability among *Xcm* isolates. **b** Principal coordinate analysis (PCoA) for

ISSR-PCR. Coordinates 1, 2, and 3 revealed 74.9% variability among *Xcm* isolates

In the present study, none of the isolates were highly virulent in the south zone except isolate KDS from Karnataka. Two isolates were highly virulent among five in the north zone. In the central zone, four isolates were highly virulent. It clearly indicated the favourable climatic conditions for disease development and severity in the central zone and north zone. In the present investigation, 62% of

the total isolates (21) recorded as race 18. Seven isolates (70%) from Maharashtra recorded as race 18. Gholve et al. (2005) reported that 88.9% isolates were race 18 among the 27 *Xcm* isolates from Maharashtra. Meshram et al. (2002) recorded 14 different races from the cotton-growing regions of India. But in the present study, only eight

different races were observed from three cotton-growing zones of India.

Even though genomic fingerprinting exhibited lot of variations, grouping of the isolates in clusters was not uniform or following any type of pattern except the location of collection. *X.campestris* pv. *campestris* isolates of brassica from Tanzania had been grouped based on the geographical location of the isolates (Massomo et al. 2003). Samanta and Mandal (2014) found that the molecular variability among 43 *X. axonopodis* pv. *commiphore* strains of Guggal from Gujarat was partially due to the location of origin of isolates. Adaptability might be the reason for these types of clustering (Massomo et al. 2003).

The Indian *Xcm* isolates have complex genetic diversity and lot of variations have been observed at the virulence level. The polymorphism may be due to nucleotide modification, insertion, and deletion at initiation sites. It might have occurred due to unfavourable biotic and abiotic stresses leading to genetic variability (Abdo-Hasan et al. 2008). Clusters and sub-clusters formed through rep-PCR were associated with the geographical origin of the isolates for *P. savastonii* pv. *savastonii* (Moretti et al. 2017). In contrast, it is extremely difficult to assign clusters based on the virulence or race of the isolates. The PC-CICR isolate used as positive control in the study was grouped with ANR (race 18 and MV) isolate from Andhra Pradesh in REP, ERIC, and combined repetitive element analyses and exhibited shared homology of 81.5%, 90%, and 79%, respectively. The PC-CICR also grouped with TDJ (race 3 and LV) and MRN (race 8 and LV) isolates in BOX by sharing a homology of 100%. In ISSR analysis, it clustered with AGD (race 11 and LV) and KDS (race 18 and HV). The PC-CICR isolate is highly virulent, belonged to race 18 and mostly clustered with less or moderately virulent isolates. This revealed the role of climatic factors and geographical origin in the virulence and genetic make-up of *Xcm* isolates.

No correlation observed between virulence and race with genomic finger printing of the *Xcm* isolates collected from various cotton growing regions of India. Valverde et al. (2007) characterized *X. campestris* pv. *campestris* isolates through REP-PCR, PFGE, and AFLP fingerprinting techniques, but could not get any clear correlation between pathogenicity and genetic diversity. They also reported that single mutations in bacterial avirulence (*avr*) genes were often responsible for the development of new races or switching of races. These were rarely identified by DNA fingerprinting techniques. Apart from that the plant secretome analysis during the pathogen infection through proteomic techniques helps to understand plant–pathogen interactions (Imam et al. 2017). In natural conditions, several abiotic stress causing factors make the plant more susceptible to pathogen infection or induce tolerance to pathogen. These were coordinated

by various biomolecules through different metabolic pathways in multifaceted interactions (Dangi et al. 2018).

Moretti et al. (2017) recorded a total variability of 48.25% among 124 *P. savastanoi* pv. *savastanoi* isolates under PCoA analysis for three coordinates. We observed 76.4% and 77.5% of total variability for PCoA analysis for combined repetitive elements and ISSR analyses, respectively. Contrary to our results, Nunes et al. (2009) observed less than 30% variation among 61 *Xcm* isolates from Brazil through combined REP and ERIC-PCR analyses.

This study established profound heterogeneity among *Xcm* isolates from three cotton-growing zones of India. A lot of intra-pathovar polymorphism has also been observed among *Xcm* isolates in India. This might be due to the spread of the pathogen races through the transport of germplasm lines and seed materials from one place to another. The present study results appear to be one of the first elaborate studies on the virulence and genomic fingerprinting of the *Xcm* isolates from India.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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