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RESEARCH ARTICLE

Genetic fingerprinting distinguishes pathogenic variability of asiatic citrus canker pathogen, *Xanthomonas citri* pv. *citri* type A

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Abstract

Citrus canker, caused by *Xanthomonas citri* pv. *citri* (Xcc), is one of the most widespread citrus disease. In this study, 54 isolates of Xcc were obtained from citrus canker-infected leaves, twigs and fruit samples collected from various citrus-growing regions in Bangladesh. These isolates were analysed through pathogenicity tests and PCR amplification. The results revealed varying levels of aggressiveness among the isolates, ranging from weak to moderate and aggressive. No correlation was found between the host plants and their geographical locations. The genetic analysis of *Xanthomonas* isolates from around the world revealed 100 % similarity. All the *Xanthomonas* isolates were grouped into 2 major clusters. DNA fingerprinting showed high genetic variability, with no correlation between hosts, geographic locations or aggressiveness levels. This information on the population diversity of the citrus canker pathogen, *Xanthomonas citri* pv. *citri* (Xcc), will be valuable for developing sustainable management strategies for citrus cankers in Bangladesh and the broader Indian sub-continent region.

Keywords

citrus canker; *Xanthomonas citri*; pathotype A; pathogenicity; aggressiveness

Introduction

Citrus species are cultivated as valuable commercial fruit crops in over 140 countries worldwide. The major citrus fruits grown globally include oranges (*Citrus sinensis*), grapefruits (*Citrus paradisi*), lemons (*Citrus limon*), limes (*Citrus aurantifolia*), mandarins (*Citrus reticulata*) and pummelos (*Citrus maxima*) (Citrus Fruit Statistical Compendium) (1). These fruits belong to the Rutaceae family and were first introduced in Australia, New Caledonia and New Guinea (2). Citrus can grow in a wide variety of soils around the world, including in Bangladesh, although they thrive best in light loam soils with good drainage and a pH range of 4 to 9. A subtropical climate is ideal for citrus cultivation, with the optimal soil temperature for root growth being approximately 25 °C. Citrus trees also benefit from annual rainfall between 75 and 125 cm and dry weather with well-defined summers is most favourable for fruit production. In 2019, global citrus production was estimated at 143 million tonnes, grown across 9.8 million ha, making it the top-ranked fruit in the world in terms of total output (3, 4). In Bangladesh, citrus is grown on a small scale in both private orchards and commercial

plantations. Between 2017 and 2018, the country produced a total of 23 million tonnes of citrus (5).

Citrus canker, caused by *Xanthomonas citri* pv. *citri* (Xcc), significantly affects citrus-growing regions worldwide, including the Indian subcontinent. The disease is characterized by corky, necrotic lesions with chlorotic halos on leaves, young stems and fruits. It impacts the overall health of the plant and reduces fruit quality, thus lowering marketability. In severe cases, it can lead to dieback and premature fruit drop. Accurate identification and classification of bacteria are essential in environmental, industrial, medical and agricultural microbiology. Various phenotypic and genotypic methods are currently employed for microbial identification and classification (6) and several molecular tools have been used to determine the population structure of *X. citri* pv. *citri.* Among these, DNA-based methods are increasingly recognized as reliable, simple and cost-effective for identifying and classifying microbes. Traditional, bacterial genera and species have been assigned using DNA-DNA hybridization techniques (7). However, modern phylogenetic studies rely more on the sequencing of 16S rDNA (8, 9). For years, various approaches have been used to identify pathogens and analyse genetic diversity. A study developed the ITS region of 16S-23S to detect Xcc (10). Additionally, bacterial fingerprinting techniques, such as repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX elements, which are present in all prokaryotes, have been employed to study the genetic diversity of Xcc (11).

Controlling citrus canker is essential to produce export-quality citrus in Bangladesh. However, managing canker, particularly in susceptible cultivars under conditions favorable for disease development, is generally challenging. The most effective approach involves the use of resistant cultivars combined with an integrated system of compatible cultural practices and phytosanitary measures, including quarantine and regulatory programs (12). Additionally, chemicals, plant extracts and biological antagonists can be used to manage citrus canker. Although chemicals are the easiest method for controlling pre- and post-harvest losses, they can contribute to environmental pollution (13). Beyond chemical approaches, both chemical and non-chemical strategies are used in other countries to reduce the spread of the disease and its pathogen (14, 15). Unfortunately, in Bangladesh, there is still no molecular data on the detection of pathotype A and or the genetic diversity analysis of *Xanthomonas citri* pv. *citri* (Xcc). Existing studies focus only on incidence, severity, epidemiology and risk analysis of citrus canker (16, 17). More than 18 *Citrus* species are cultivated in Bangladesh, with citrus canker posing a major threat to most species except Pummelo (*C. maxima*) Batabi lebu; Mandarin (*Citrus reticulata*) and some citrons. Currently, Bangladesh exports Jara lemons produced in the greater Sylhet region and Colombo lemons mainly from Narsingdi, generating 75 crore BDT in the current fiscal year (Source: Plant Protection Wing, DAE). However, the country still faces significant challenges in citrus exports due to citrus canker, which has reduced citrus exports by approximately 50 %. At one point, the export of Jara lemons (*Citrus medica*) to Europe was halted, creating a national concern for the citrus export industry. Understanding the population structure of the pathogen, alongside the mechanisms driving genetic changes within the pathogen population, is crucial for developing long-term disease management strategies (18). In this study, we collected 54 isolates of Xcc from different citrus-growing regions of Bangladesh. We assessed the genetic diversity of these Xcc strains using 16S-rDNA and rep-PCR from symptomatic citrus trees and compared them to global collections to identify possible geographic origins and genetic variations of Xcc isolates.

Materials and Methods

Sampling

During the survey, leaves, twigs and fruits showing typical canker symptoms were collected based on visual observation (19), from various citrus-growing regions in Bangladesh (Table 1). To assess disease incidence, 5 trees were randomly selected in each host, and 40 leaves per plant were examined for disease severity. Canker-infected samples were transported to the Laboratory in sealed ballooned Zip bags following standard procedure. The samples were then stored in a refrigerator at 4 °C for isolation of the causal bacterium and subsequent laboratory analyses.

Isolation, purification and conservation of the causal bacterium

Canker-infected samples (leaves, twigs and fruits) showing typical symptoms were thoroughly washed repeatedly with tap water. Small pieces of tissue were then cut from a young lesion or the margin of older lesions using a sharp, sterilized blade or scalpel and surface-sterilized with 70 % ethanol. After rinsing with sterilized distilled water, the tissue was placed in Eppendorf tubes containing 1 mL of sterilized distilled water and macerated using forceps or a needle. The tissues were kept in suspension for at least 30 min to allow the bacterium to diffuse out. The resulting suspension was streaked onto plates of Yeast Peptone Sucrose Agar (YPSA) medium (Yeast extract 10 g, Peptone 20 g, Sucrose 20 g and Agar 20 g medium) containing cycloheximide (200 mg/L) with an inoculating loop. The plates were incubated at 28 ± 2 °C for 2-3 days. Pure bacterial cultures were maintained in LB liquid media at -80 °C for long-term storage.

Inoculum preparation

Bacterial growth observed on the plates was recorded, and colonies exhibiting the typical morphological characteristics of *X. citri* pv. *citri* were selected using a sterilized loop. Purified cultures were obtained by streaking these colonies on YPSA media. All inoculated plates were incubated at 28 °C for 48-72 h to allow the growth of the bacteria. The bacterial cells were then suspended in sterile distilled water and the bacterial suspension was adjusted to a concentration of 10^8 CFU/mL (OD660=0.3) (20).

Detached leaves pathogenicity assay

The *in vitro* pathogenicity test using a detached leaf assay was conducted following the method (21). A single sterilized leaf was placed in a petri plate with its abaxial surface facing up. The leaf was inoculated at 6 different points using a syringe to apply10 µL of bacterial suspension containing approximately 10^8 CFU/mL. The puncture points were made with a sterile pin and the petri dish was sealed with a lid lined with moist blotter paper. In the control group, a pin dipped only in sterilized water was used. The petri plates were placed in a growth chamber at 28 ± 2 °C with a photoperiod of 12 h of light and 12 h of darkness for 3 weeks. Symptoms were observed and recorded and the isolates were categorized into different pathogenic groups based on their aggressiveness.

In *planta* Pathogenicity test

The protocol followed was based on a study (22). Fully expanded leaves were infiltrated by gently pressing the syringe aperture, without a needle, onto the abaxial leaf surface while supporting it with a finger. The leaves were inoculated with a bacterial suspension of *Xanthomonas citri* pv. *citri* (Xcc) strains at a concentration of 10^8 CFU/mL. The plants were maintained in a greenhouse at a temperature of 28 and 30 °C.

Extraction of genomic DNA from *X. citri* pv. *citri*

The genomic DNA of *Xanthomonas citri* pv. *citri* was extracted using the Wizard DNA Kit, following the manufacturer's instructions in the Plant Bacteriology and Biotechnology Laboratory at Bangladesh Agricultural University. The procedure involved incubating a single colony of *X. citri* pv. *citri* for 24 h at 28 ± 2 °C, then transferring a loopful of the bacterial culture to YPSA broth and pelleting the cells. The supernatant was discarded and a nucleus lysis solution was added. The mixture was incubated at 80 °C for 5 min, then cooled to room temperature. RNase A solution was added and the mixture was incubated at 37 °C for 15-60 min. A protein precipitation solution was added and the mixture was incubated on ice for 5 min before centrifuging at 12000 rpm for 3 min. The clear supernatant was transferred to a clean tube containing 600 µL of room-temperature isopropanol and the mixture was centrifuged at 12000 rpm for 3 min. The remaining ethanol was aspirated and the pellet was air-dried for 10-15 min. The DNA pellet was then rehydrated with 100 µL of DNA rehydration solution (TE) and left overnight at 4 °C. The genomic DNA samples from all isolates were stored at -20 °C for further use.

16S rDNA identification of *Xanthomonas* strains

For the genetic diversity analysis, amplification reactions were performed using 16S rDNA primer sets: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1518 R (5'AAGGAGGTGATCCANCCR CA-3'). Species-specific primers J-RXg/J-RXc2 were used to identify the pathotype of Xcc strains tested in this study (10).The primer sequences were J-RXg (5′-GCGTTGAGGCTGAGACATG) and J-RXc2 (5′-CAAGTTGCCTCGGAGCTATC).

16S rDNA sequence analysis

Eight representative isolates of Xcc were selected based on *in vitro* and *in vivo* pathogenicity tests and amplified at 1500 bp using 16S primer sets. Sequencing of the PCR products, amplified with the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), was performed using an ABI 3730xl DNA genetic analyzer at Macrogen, South Korea. Twenty-seven *Xanthomonas* sequences from global isolates were collected from the NCBI database to assess phylogenetic relationships. Similarities were calculated using the maximum phylogenetic relationship with 500 bootstraps in the MEGA version 11 program.

PCR amplification for genomic fingerprinting

For genetic diversity analysis, amplification reactions were conducted using 16S rDNA primer sets:27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1518R (5'-AAGGAGGTGATCCANCCR CA-3') along with 3 rep-PCR primersets: REP1(IIIICGICGICATCIGGC), REP2 (ICGICTTATTATCIGG CCAC); ERIC1 (ATGTAAGCTCCTGGGGATTCAC), ERIC2 (AAGTAAGTGAC TGGGGTGAGCG) and BOX-PCR (CTACGGCAAGGCGACGCTGACG). For rep-PCR,1 µL template DNA was used per reaction. Amplification was performed in a BIORAD C1000TM thermocycler. The protocol began with a denaturation stage at 95°C for 7 min, followed by 35 cycles of 94 °C for 1 min, 44 °C for 1 min and 65 °C for 15 min (21). The amplification conditions for ERIC primers included 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min, with an initial step at 95 °C for 7 min and a final extension at 65 °C for 15 min (21). For BOX primers, the cycling program started with denaturation at 95 °C for 7 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 8 min at 65 °C for primer extension (23).The DNA from the REP, ERIC and BOX PCR products were analysed using 1.5 % agarose gel electrophoresis in 1XTBE buffer at 90V for 2 h and stained with ethidium bromide.

Genomic fingerprinting and Cluster analysis

The Fingerprinting results from REP, ERIC and BOX PCR were converted into binary form (0 = absence or negative; 1 = presence or positive). Data analysis was conducted using NTSYS software (Exeter Software, Setauket, NY) to perform phylogenetic analyses and assess the relationships among strains collected from different growing regions and hosts. Three primer sets were used to analyze the combined and clustered data. The dice coefficient was employed to calculate similarities and clustering was performed using the UPGMA's NTSYS version 2.1 (Exeter Software Setauket, NY).

Results

Identification of Asiatic citrus canker pathogen type A *Xanthomonas citri* pv. *citri* by PCR amplification and pathogenicity test on a wide host range

From canker-infected citrus leaves and fruits collected from various hosts and locations, 54 bacterial isolates were identified (Table 1). A PCR-based approach was employed to identify *Xanthomonas citri* pv. *citri*

pathotype A (24). This study used a highly specific set of primers (J-RXg and J-RXc2) to detect Xcc. The amplicon size of 179 bp confirmed that all the isolates obtained from different canker-infected plant samples collected from various locations were *Xanthomonas citri* pv. *citri,* type A, the pathogen responsible for Asiatic citrus canker (Fig. 1).

The study also analyzed the responses of different citrus hosts to *Xanthomonas citri* pv. *citri* isolates collected from seven different citrus-growing regions. Pathogenicity tests were conducted on 5 different hosts and the results revealed that all isolates produced symptoms on the leaves (Table 1). The isolates were categorized based on their aggressiveness as Aggressive (A), Moderately Aggressive (MA) and Weakly Aggressive (WA), depending on the severity of symptoms observed.

Among the 5 host studies, 22.22 % of isolates from lime showed aggressive behaviour, 20.37 % were moderately aggressive and 57.40 % were weakly aggressive (Fig. 1). In lemon, 7.41 % of isolates were moderately aggressive, 92.59 % were weakly aggressive and 3.70 % were aggressive. For mandarin, 20.37 % of isolates were moderately aggressive and 75.92 % were weakly aggressive. Sweet orange isolates showed 18.51 % aggressive, 35.18 % moderately aggressive and 46.29 %

Table 1. Aggressiveness of *Xanthomonas citri* pv. *citri* isolates on different citrus hosts as assessed by the pathogenicity test.

Isolate name	Symptoms severity				
	Lime *(Citrus aurantifolia)*	Lemon (*Citrus limon*)	Mandarin (*Citrus reticulata*)	Sweet orange (*Citrus sinensis*)	Pummelo (*Citrus grandis*)
XacCM6S1	WA	WA	WA	A	WA
XacBM2S2	WA	WA	WA	WA	WA
XacVM1K1	A	WA	WA	WA	WA
XacJL4M1	WA	WA	MA	A	WA
XacSL5S3	MA	WA	WA	MA	WA
XacKLM1K2	WA	WA	MA	WA	WA
XacSL1M2	MA	WA	WA	A	WA
XacBM1M3	MA	WA	WA	WA	WA
XacKLM3K3	A	WA	A	WA	WA
XacKL1M4	WA	WA	MA	A	MA
XacJL1M5	A	WA	WA	A	WA
XacSL5M6	WA	WA	WA	MA	MA
XacKL3M7	WA	WA	MA	MA	A
XacBM1K4	WA	WA	WA	MA	MA
XacBL1JA1	A	WA	WA	WA	MA
XacVMK5	MA	WA	WA	MA	WA
XacKL3TA1	MA	WA	MA	A	A
XacLL6S4	MA	WA	WA	WA	A
XacKL1TA2	MA	WA	WA	A	MA
XacBM1S5	WA	WA	WA	A	A
XacCM10S6	WA	WA	MA	A	MA
XacJL7S7	WA	WA	WA	WA	WA
XacJL2S8	WA	WA	MA	MA	MA
XacBMK10K6	MA	WA	WA	WA	MA
XacSL1JA2	WA	WA	WA	MA	MA
XacJL4JA3	WA	WA	WA	A	MA
XacBM3K7	A	WA	MA	MA	MA
XacCMJ2JA4	MA	WA	WA	MA	WA
XacCMJ1JA5	WA	WA	MA	MA	WA
XacCRJA1JA6	WA	WA	WA	WA	WA
XacBL3JA7	WA	WA	WA	MA	WA
XacJL10S9	WA	WA	WA	WA	WA
XacLL3S10	WA	WA	WA	WA	A
XacKLM1JA8	WA	WA	MA	WA	MA
XacCLJA4JA9	WA	WA	WA	WA	MA
XacBL3JA10	WA	WA	WA	WA	MA
XacLL9S11	WA	WA	WA	WA	MA
XacCLJA1JA11	WA	WA	WA	WA	WA
XacCLJA3JA12	WA	MA	WA	WA	MA
XacKL5My1	WA	MA	WA	WA	MA
XacSA1M8	WA	WA	WA	WA	WA
XacBK2M9	A	MA	WA	MA	MA
XacKLM1M10	A	WA	WA	WA	MA
XacJL6M11	WA	WA	WA	WA	MA
XacBK1K8	MA	WA	WA	WA	WA
XacBK2K9	A	WA	WA	WA	MA
XacBK6K10	A	MA	MA	MA	MA
XacKL2JA13	A	WA	A	MA	MA
XacKL5JA14	A	WA	WA	MA	MA
XacKL3My2	MA	WA	WA	MA	WA
XacBM1M12	WA	WA	WA	WA	WA
XacKLM10M13	WA	WA	WA	MA	WA
XacKLM7M14	WA	WA	WA	MA	MA
XacKLM7Kha11	A	WA	WA	MA	MA
Control	-	-	-	-	--

Aggressive, forming large necrotic or wet areas (A), moderately aggressive, producing a small volume of necrotic regions surrounding the lesion and a water-soaking area of less than 1 mm around the lesion (MA), weakly aggressive, creating very few necrotic regions and/or water-soaking areas around the lesion (WA).

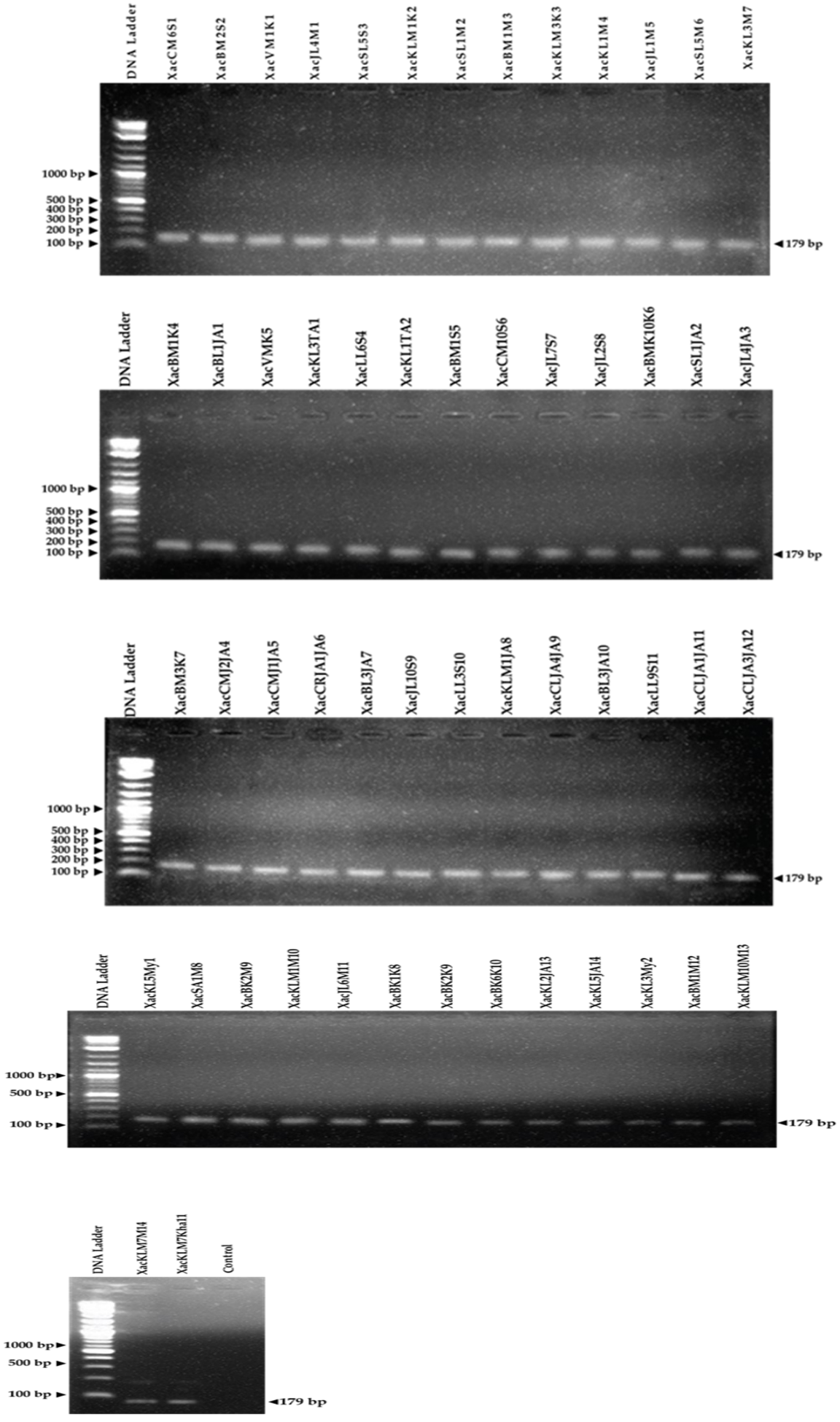

Fig. 1. Identification of *X. citri* pv. *citri* by PCR utilizing primers (*J-RXg* and *J-RXc2*) and genomic DNA template.

weakly aggressive responses. In pummelo, 9.26 % of isolates were aggressive, 48.15 % moderately aggressive and 42.59 % weakly aggressive. This result suggests that *Xanthomonas citri* pv. *citri.* Pathotype A isolates exhibit varying degrees of aggressiveness depending on the host, with the bacterial isolates collected from different hosts showing diverse pathogenicity.

Pathogenic groups of *X. citri* pv. *citri* considering both detached leaf and in *planta* pathogenicity test

A total of 54 isolates of the citrus canker bacterium were obtained from different hosts, including BARI kagoji labu-1, Jara lemon, Seedless lemon, Colombo lemon, BARI Malta-1, Local lemon, Batabi lemon, BARI Komola, Satkora and others. The pathogenic variability among these 54 isolates of *Xanthomonas citri* pv. *citri* on citrus leaves was studied (Table 2). All isolates were found to be susceptible to canker, exhibiting diverse reactions in symptom development. The classification of the isolates was based on the appearance of symptoms on leaves used for the pathogenicity test. Regarding pathogenic virulence on detached leaf, 12.96 % of the isolates were categorized as aggressive, 48.15 % as moderately aggressive and 38.88 % as weakly aggressive. For virulence in *planta* leaves, 3.70 % of the isolates were aggressive, 14.81 % were moderately aggressive and 81.48 % were weakly aggressive.

A combined analysis of the 54 isolates, based on aggressiveness was performed irrespective of their locations and hosts. Cluster II included 2 aggressive isolate from Sylhet and Khagrachari. Additionally, Cluster III and Cluster I comprised 18 and 34 isolates respectively. The combined pathogenicity test on both detached and in *planta* leaves resulted in 2 aggressive isolates (3.71 %), 34 moderately aggressive isolates (62.96 %) and 18 weakly aggressive isolates (33.33 %) among the total 54 isolates. No relationship between the host plants and the locations of the isolates was observed based on their aggressiveness (Fig. 2).

Table 2. Aggressiveness of different isolates on detached leaf and in *planta* leaf.

Isolate name	Virulence on detached leaf	Virulence on in *planta* leaf
XacCM6S1	WA	MA
XacBM2S2	A	A
XacVM1K1	MA	WA
XacJL4M1	A	WA
XacSL5S3	A	WA
XacKLM1K2	A	WA
XacSL1M2	MA	WA
XacBM1M3	WA	WA
XacKLM3K3	WA	WA
XacKL1M4	A	WA
XacJL1M5	A	WA
XacSL5M6	MA	WA
XacKL3M7	MA	MA
XacBM1K4	A	A

Aggressive, forming large necrotic or wet areas (A), moderately aggressive, producing a small volume of necrotic regions surrounding the lesion and a water-soaking area of less than 1 mm around the lesion (MA), weakly aggressive, creating very few necrotic regions and/or water-soaking areas around the lesion (WA).

Genetic variability analysis of 54 isolates of *Xanthomonas citri* pv. *citri* using 16S rDNA primers

Eight isolates of *X. axonopodis* pv. *citri* , which displayed the most severe symptoms on Kagaji lemon (Lime), were selected to examine the genetic diversity of *Xanthomonas* strains in comparison to global isolates using 16S rDNA analysis. All isolates were amplified at 1500 bp and sequenced, resulting in the classification of 35 isolates into 2 major clusters. Seven out of the eight isolates from Bangladesh formed a single cluster, while the isolate XacKL1M4 grouped with global isolates in the phylogenetic analysis. All of these *Xanthomonascitri* pv. *citri* isolates exhibited 100 % similarity with the global isolates (Fig. 3).

Genomic fingerprinting of 54 isolates of *Xanthomonas citri* pv. *citri* using rep-PCR

The genetic diversity of 54 Xcc isolates was assessed using rep-PCR. The REP, ERIC and BOX primers generated 21, 23 and 14 bands respectively, all of which (100 %) were considered polymorphic.

Genomic fingerprinting using REP primer

All rep-PCR products from Xcc demonstrated banding patterns with sizes ranging from approximately 100 to 1600 bp. Cluster analysis based on a 70 % similarity coefficient, revealed three major clusters. Cluster I included 46 isolates from various hosts (sweet orange, pummelo and lemon) and locations (Sylhet, Khagrachari, Jamalpur, Tangail and Moulvibazar), with varying levels of aggressiveness. Cluster II contained 7 isolates from Moulvibazar, Jamalpur, Khagrachari and Mymensingh. Cluster III comprised a single isolate from citron, collected in Moulvibazar (Fig. 2).

Genomic fingerprinting using ERIC primer

For the ERIC primers, the isolates were differentiated into 10 different clusters. Cluster I contained 6 isolates from Sylhet, Khagrachari, Jamalpur and Moulovibazar. Cluster II, the largest cluster, consisted of 23 isolates from Sylhet, Khagrachari, Jamalpur and Moulovibazar. Cluster III included 1 isolate from Sylhet. Cluster IV comprised 3 isolates from Sylhet, Khagrachari and Moulovibazar, while cluster V had 1 isolate of Jamalpur. Cluster VI contained 5 isolates from Jamalpur, Mymensingh and Moulovibazar. Cluster VII included 6 isolates from Khagrachari and Moulovibazar. Cluster VIII comprised 3 isolates from Jamalpur, Mymensingh and Moulovibazar, while cluster IX included 4 isolates from Jamalpur and Khagrachari. Finally, Cluster X contained 1 isolate from Sylhet (Fig. 3).

Genomic fingerprinting using BOX primer

Seven major clusters were identified from the BOX AIR PCR analysis. Cluster I, the largest, included 32 isolates from Sylhet, Khagrachari, Jamalpur, Tangail and Moulovibazar. Cluster II contained 2 isolates from Sylhet and Jamalpur, while Cluster III comprised of 10 isolates of Sylhet, Jamalpur and Khagrachari. Cluster IV had 2 isolates from Sylhet. Cluster V included 1 isolate from Sylhet and cluster VI also contained 1 isolate from Sylhet. Finally, cluster VII consisted of 5 isolates from Jamalpur, Mymensingh and Moulovibazar (Fig. 4). Variation was observed based on

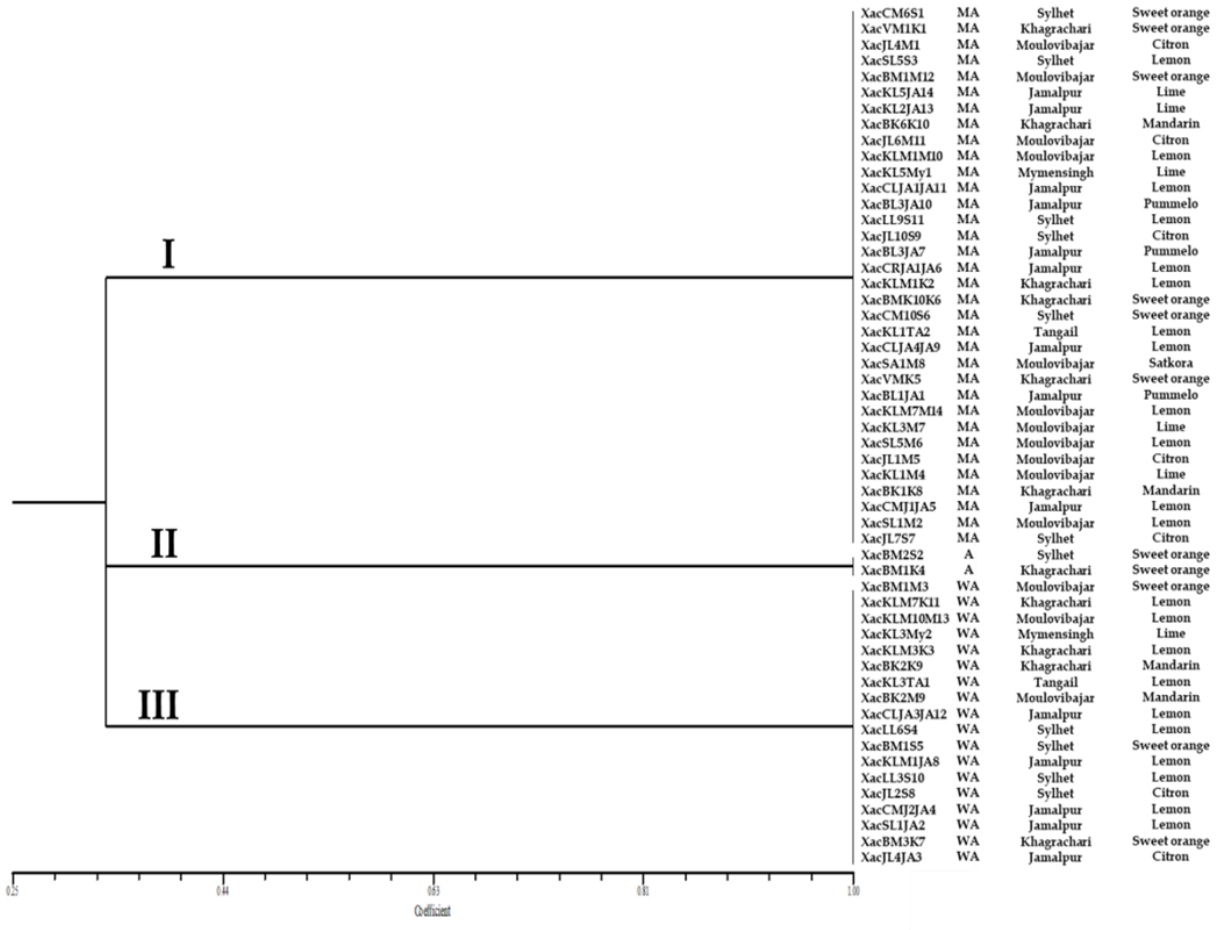

Fig. 2. Dendrogram showing pathogenic relationship among 54 isolates of *Xanthomonas citri* pv. *citri* on artificially inoculated both detached and in *planta* leaves of BARI kagoji lebu 1 by using the dice coefficient and clustering was achieved by UPGMA using the NTSYS version 2.1.

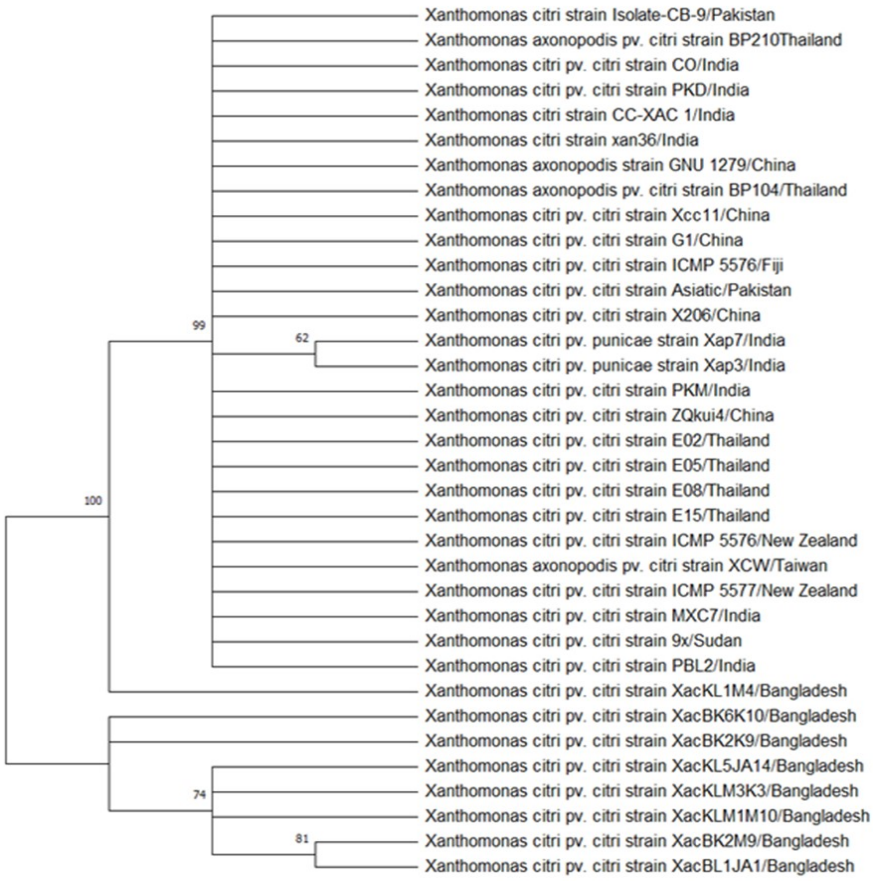

Fig. 3. Dendrogram showing the phylogenetic relationship of 35 *Xanthomonas* isolates from worldwide on 16s rDNA PCR analysis. The highest phylogenetic connection was used to calculate similarities in 500 bootstraps MEGA version 11 program.

locations and hosts. A combined cluster analysis of the 54 isolates revealed a total of 24 different clusters, each containing between 1 to 6 isolates. Cluster V emerged as the largest, comprising 6 isolates from Khagrachari and Tangail. This is followed by clusters I, III, IV and VII, each containing 4 isolates from various locations. The remaining clusters are smaller, consisting of 1-3 isolates from Mymensingh, Jamalpur, Moulovibazar, Sylhet and Khagrachari (Fig. 4). Overall, the rep-PCR and combined cluster analysis indicated varying relationships between aggressiveness, hosts and location.

Discussion

Xanthomonas citri pv. *citri*, which causes citrus canker, is one of the most damaging diseases affecting citrus-growing regions worldwide, including the Indian subcontinent. This disease significantly impacts plant health and fruit quality, ultimately reducing the market value of citrus products. It is characterized by erumpent corky necrotic lesions surrounded by a chlorotic halo on leaves, young stems and fruits. In severe cases, symptoms may include black spots, defoliation, rupture of the leaf epidermis, decreased photosynthetic rates, dieback and premature fruit drop (19).

A total of 54 different isolates were obtained from various hosts, including BARI Kagoji lebu-1, Jara lemon, Seedless lemon, Colombo lemon, BARI Malta-1, Local lemon, BARI Komola, Satkora, Batabi lemon and Variegated Malta. A representative isolate was selected for pathogenicity testing, which was conducted on BARI Kagoji lebu-1 using both detached and in *planta* leaves. The results indicated that all *Xanthomonas citri* pv. *citri* isolates had the capability to cause infection, as demonstrated by the absence of canker (-) as well as weakly aggressive (WA), moderately aggressive (MA) and aggressive (A) signs. Based on their aggressiveness towards BARI Kagoji lebu-1, the 54 isolates were categorized into 3 major clusters: Cluster I, which included 34 moderately aggressive isolates; Cluster II, which contained 2 aggressive isolates and Cluster III, which comprised 18 weakly aggressive isolates. Notably, no pathogenic relationship was observed between the isolates and their hosts or locations. A study reported similar findings based on virulence profile analysis (25), noting no observable correlations between race, host or country of origin, which highlights the high levels of diversity of *X. campestris* pv. *campestris* in Portugal. Additionally, another study found no association between virulence variation and ecological zones, despite the distribution of isolates with varying levels of aggression across different ecological zones (26).

The PCR approach for detecting *Xanthomonas citri* pv. *citri* has been demonstrated by several researchers both nationally and internationally. A study noted that serology, along with PCR and DNA analysis, can be used for

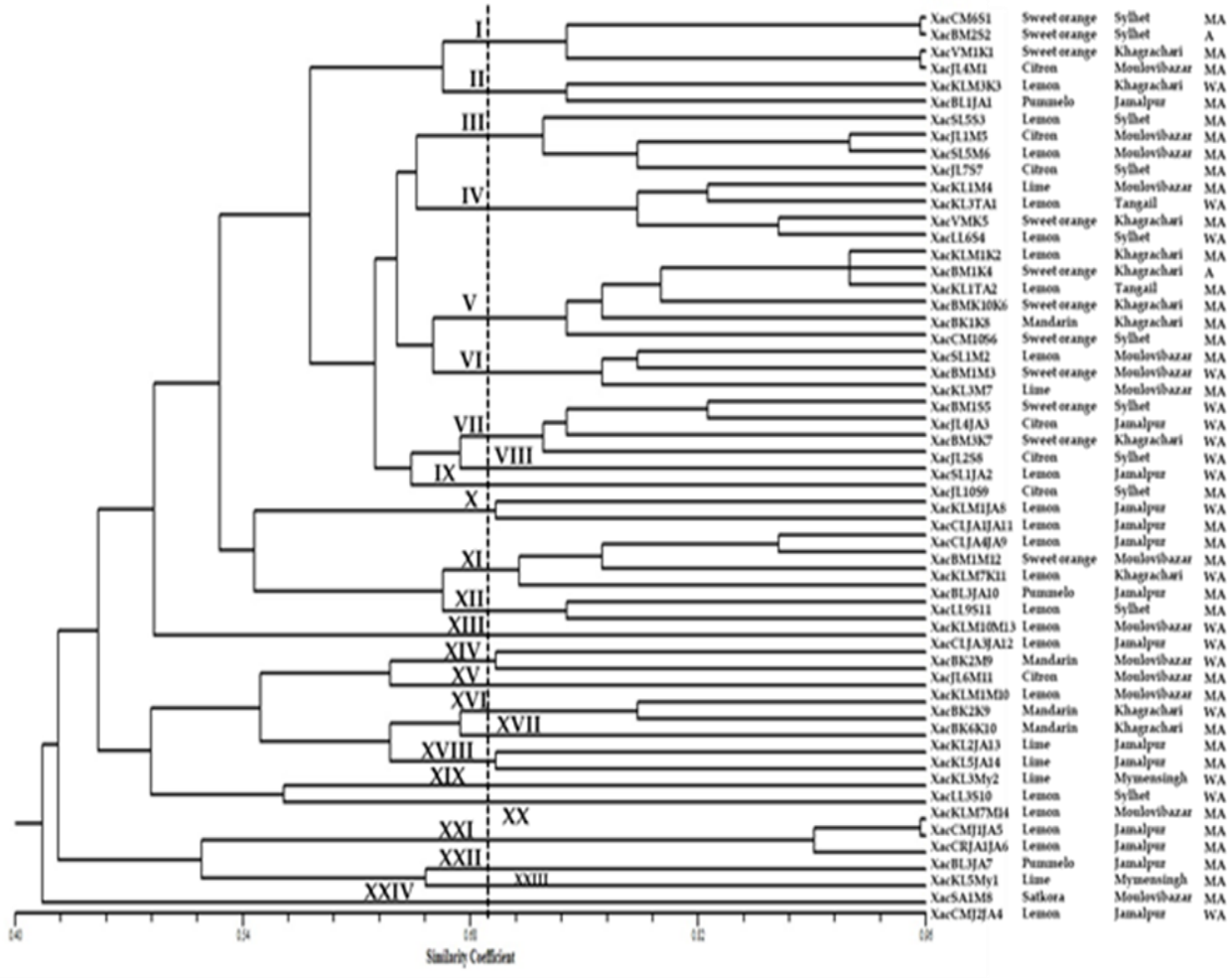

Fig. 4. Dendrogram showing relationships among *X. citri* pv. *citri* isolates causing CBC based on REP, ERIC and BOX PCR combined analysis. Similarities were calculated by using the dice coefficient and clustering was achieved by UPGMA using the NTSYS version 2.1 and MEGA version 11 programs.

the identification and pathovar categorization of bacterial isolates (10). While plasmid-based primers (*J-pth1* and *J-pth2*) are effective and stable, they cannot detect strains of Xcc. Consequently, another study developed specific primers (*J-RXg* and *J-RXc2*) based on the variable ITS region of the 16-23S rDNAs (10). These primers were designed to amplify specific DNA from *X. citri* pv. *citri,* J-RXc2 exclusively annealed to DNA from *X. citri* pv. *citri* strains, while J-RXg annealed with DNA from all the *Xanthomonas* strains studied. In this research, the canker-causing *Xanthomonas citri* pv. *citri* was identified by PCR using the primers J-RXg and J-RXc2, which amplified a fragment of 179 bp.

Citrus canker outbreak affects all citrus-growing regions worldwide and our survey observed extensive spread. However, there has been a lack of comprehensive genomic fingerprinting of Bangladeshi isolates based on different hosts and growing areas. A genetic diversity study using repetitive elements (REP, ERIC, BOX) and 16s rDNA on 54 Bangladeshi Xcc isolates revealed a variety of genetic variants. The rep-PCR technique is widely used to examine diversity among various prokaryotic organisms (27), particularly for gram-negative bacteria like *Xanthomonas* (28), *Ralstonia* (29), *Pseudomonas* (30) and *Agrobacterium* (31), making it a reliable choice for this study.

In the analysis of the partial 16S rDNA sequences, 8 isolates of Xcc from the most susceptible host, Kagazi lebu, demonstrated a significant relationship with global isolates, showing 100 % similarity. All *Xanthomonas citri* pv. *citri* isolates were categorized into 2 major clusters. Seven out of the eight Bangladeshi isolates (XacBL1JA1, XacKL5JA14, XacKLM3K3, XacBK2K9, XacBK6K10, XacBK2M9 and XacKLM1M10) formed a separate cluster, while the remaining isolate (XacKL1M4) joined the global isolates in a larger cluster. This suggests that Xcc may have been introduced into our country through the migration of planting materials. A study noted that after the Xcc was eradicated from South Africa in 1938 (32, 33), the migration of planting materials to commercialize citrus - following the loss of cash crops-was a major factor in the invasion of Xcc in Mali and Burkina Faso. The rep-PCR analysis resulted in different clustering patterns: 4, 10, 7 and 24 clusters were observed using REP, ERIC, BOX and combined methods respectively, for the 54 isolates from 6 different hosts and 7 different regions of Bangladesh. The variability in the banding patterns of rep-PCR indicates differences in the conserved regions among these isolates. In the combined cluster analysis, using a 70 % similarity coefficient, no correlation was found between the hosts and geographic regions. Similar results were reported who found no links between cultivar, geographic area or year of origin in their combined examinations of rep-PCR among Brazilian *X. campestris* pv. *viticola* strains and *X. campestris* pv. *campestris* races (34, 35).

The BOX element in *Xanthomonas axonopodis* pv. *citri* (Xcc) was identified as the most conserved region for detecting polymorphism, yielding 14 polymorphic bands (11). In contrast, ERIC-PCR exhibited the highest degree of polymorphism, surpassing both REP and BOX elements. The genetic variability observed in Xcc may result from the introduction of different cultivars across various hosts and mutations of the pathogen influenced by agroclimatic conditions. This conclusion is supported by the findings (36) in *X. citri* pv. *malvacearum* from India, in *X. campestris* pv. *campestris* from Nepal (37), in *X. axonopodis* pv. *phaseoli* from Spain (38) and in *X. campestris* pv. *viticola* from Brazil (34). The movement of genetic lines and seed materials may also contribute to the heterogeneity observed in *X. citri* pv. *malvacearum* across 3 cotton-growing regions (36). The genetic diversity of Indian Xcm isolates is complex, with numerous variants at the pathogenicity level. Polymorphism may arise from insertions, deletions and nucleotide changes at start sites, potentially triggered by harmful biotic and abiotic stressors (39). A study employed REP-PCR, PFGE and AFLP fingerprinting methods to characterize *X. campestris* pv. *campestris* isolates but could not establish a direct link between pathogenicity and genetic diversity (40). The study demonstrated the diversity of Xcc isolates from seven citrus-growing regions in Bangladesh, highlighting significant intra-pathovar polymorphism. However, a whole genome analysis of Xcc would be the next step to gain precise insights into its origin in Bangladesh. Such comparative genome analysis will aid in identifying important virulence factors, such as transcription activator -like effectors (TALs). Identifying these TAL effectors would be beneficial for determining their corresponding host targets.

Conclusion

Rep-PCR can serve as a molecular marker to distinguish the molecular haplotypes of *Xanthomonas citri* pv. *citri* responsible for Asiatic canker in citrus. Further studies involving a large number of isolates are needed to provide additional insights into the genetic variation of Xcc, which could inform strain-specific management strategies in the country. Advanced sequencing technologies, such as nanopore sequencing, could facilitate more detailed discrimination of the genetic variation within the *X. citri* pv. *citri* population and help identify new components, such as nanoparticles, for managing canker in other citrus species.

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Authors' contributions

RI, IAP and ASMT conducted the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. IAP, ASMT, URS, AS and MH carried out the immunoassays. IAP, SHT, MHH and IK participated in the sequence alignment. RI, MRI, SA and ASMT participated in the study design and performed the statistical analysis. RI, SHT, MAA and MRI conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflict of interest to declare.

Ethical issues: None

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