GENETIC DIVERSITY AMONG *XANTHOMONAS CITRI* SUBSP. *CITRI* STRAINS IN IRAN

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Abstract: Citrus bacterial canker (CBC) is one of the most important diseases of citrus. It is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*). To investigate the variability of *Xcc*, a collection of twenty-five strains were isolated from the Fars, Hormozgan, Kerman and Sistan-va-Baluchestan provinces of Iran. The twenty-five strains were assessed phenotypically and genetically. These strains had similar biochemical properties. Based on host range determination, the strains were divided into two groups; the first group was pathogenic on Mexican lime (*Citrus aurantifolia*), citrumelo (*Poncirus trifoliata* x *C. paradisi*), citrange (*C. sinensis* x *P. trifoliata*) and sour orange (*C. aurantium*) varieties. The second group was pathogenic on Mexican lime only. Profile of cellular soluble proteins analyzed by sodium dodecyl sulphate-polyacryamid gel electrophoresis (SDS-PAGE) did not reveal any considerable differences among strains. Genetic diversity analyses were performed using two marker systems; repetitive polymerase chain reaction (rep-PCR) and random amplified polymorphic DNA (RAPD). The results of this research showed that two primers, ERIC 1R and 232, with the highest marker index, resulted in the most genetic variability among strains. Cluster analysis by band patterns showed that strains from the Sistan-va-Baluchestan province were a different group, so it was concluded that geographical origin of strains from the Sistan-va-Baluchestan province is different than the geographical origin of strains isolated from other provinces.

Key words: RAPD, rep-PCR, citrus bacterial canker

INTRODUCTION

Citrus bacterial canker (CBC), caused by *Xanthomonas citri* subsp *citri* (Schaad *et al.* 2006) is one of the most devastating diseases that affects many kind of commercial citrus varieties. The origin of CBC is not clear but thought to have originated from south-east Asia or India and then widely distributed around the world (Civerolo 1984; Vernière *et al*. 1998). The main symptoms of CBC are hyperplasia-type lesions on leaves, fruit and stems which in severe infections can cause leaf abscission, twig dieback and premature fruit drop (Stall and Civerolo 1991; Gottwald *et al*. 1993). The bacterium was first named as *Pseudomonas citri* (Hasse 1915). In 1939 it was classified as genus *Xanthomonas* sp. (*X. citri*) then reclassified in 1980 (Dye *et al*. 1980) as *Xanthomons campestris* pv. *citri* due to inadequate phenotypic data (Young *et al*. 1978). The bacterium has been divided in five different forms or pathotypes, A (*Xanthomonas axonopodis* pv. *citri*) B/C/ D, (*X. a*. pv. *aurantifolii*) and E (*X. a.* pv. *citrumelo*), respectively (Vauterin *et al*. 1991, 1995). Three separate taxa, *X. smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii* and *X. alfalfae* subsp. *citrumelo* were proposed by Schaad *et al.* (2005) that currently are classified as species *X. citri* ("A"), *X. fuscans* ("B/C/D") and *X. alfalfae* ("E") (Schaad *et al*. 2006). Pathotype A (Asiatic form) of CBC has a wide host range and is pathogenic on almost all citrus varieties (Vernière *et al*. 1998). There are two strain groups with a restricted host range within pathotype A. They were found in Southwest Asia, and the state of Florida in the USA, and named *X. c.* subsp. *citri* A* and A^w, respectively. The A^* strain is pathogenic only on Mexican lime (*Citrus aurantifolia*); this strain is closely related to pathotype A. The A^w strain behaves similarly, but its restricted host range includes Mexican lime and alemow (*Citrus machrophylla* Wester) (Vernière *et al*. 1998; Cubero and Graham 2002; Sun *et al*. 2004). CBC in Iran was first reported on Mexican lime trees from the Kahnouj region of the Kerman province (Alizadeh and Rahimian 1990). There are many approaches which allow discrimination of the different forms of CBC causal agent such as: physiological and serological tests, phage typing, restriction enzyme analysis, total soluble protein profile, and PCR based methods (Graham *et al*. 1990; Egel *et al*. 1991; Hartung 1992; Pruvost *et al*. 1992; Louws *et al*.1994; Vernière *et al*. 1998). Rep-PCR marker has been used for analysis of several genera as well as species of bacteria and strain identification (Louws *et al*. 1994; Cubero and Graham 2002). Primers of rep-PCR marker design are based on families of repetitive DNA sequences, repetitive extragenic palindromic (REP) sequence, entrobacterial repetitive intergenic consequence (ERIC) sequence and the BOX elements, which are present in all prokaryotes (Versalovic *et al*. 1991, 1994). Rep-PCR has been used to

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assess variation among different strains of CBC casual agent and other *Xanthomonas* species (Louws *et al*. 1994, 1995; Opgenorth *et al*. 1996; Cubero and Graham 2002; Lee *et al*. 2008;). RAPD marker is a PCR-based technique that amplifies anonymous PCR fragments from genomic template DNA, by the use of primers with an arbitrary nucleotide sequence (Williams *et al*. 1990). In bacteria, RAPDs could be a useful method to show intra-and inter-specific differences between groups of strains (Welsh and McClelland 1990).

Iranian strains of *Xcc* Isolated from the Kerman, Hormozgan and Fars provinces have been studied based on their physiological and biochemical properties (Mohammadi *et al*. 2001). Genetic diversity among these strains has been performed using AFLP marker (Khodakaramian and Swings 2002). The rep-PCR and RAPD techniques had not yet been used for Iranian strains of *Xcc*. Therefore the main purpose of this study was to examine genetic diversity among Iranian strains by rep-PCR and RAPD analysis, and to compare the discrimination power of these markers in evaluating the degree of heterogeneity in the population of *Xcc*.

MATERIALS AND METHODS

Bacterial strains

The strains of *X. citri* subsp. *citri* used in this study, were isolated from infected tissues of Mexican lime (*Citrus aurantifolia*). The lime came from the Kerman, Fars, Hormozgan and Sistan-va-Baluchestan provinces in southern Iran and had typical symptoms of CBC. One strain from the Philippines which had been accidentally imported to Iran, was also used in the study (Table 1).

Phenotypic characters

All strains were compared on the basis of their biochemical and metabolic properties as follows: gram reaction by the use of 3% KOH, oxidative/fermentative growth, nitrate reduction, oxidase reaction, hydrolysis of aesculin and gelatin, levan production, H_2S generation from cysteine, effect on litmus milk, hydrolysis of casein, and carbon source utilization which was carried out on Ayer basal medium with 1.2% agarose and final concentration 0.25% of tyndallized carbohydrate (Schaad *et al*. 2001). Hydrolysis of Tween 20 and 80, hydrolysis of starch (Fahy and Persly 1983).

Detection by polymerase chain reaction (PCR)

Bacterial DNA was extracted using the alkaline lysis method with 0.05 M NaOH (Rademarker and Debruijn 1997). PCR was conducted in a final volume of 25 µl in a thermocycler (Mastercycler® gradient). The PCR mixture contained final concentrations of 2.5 mM $MgCl_2$, 0.16 mM each of deoxyribonucleotide triphosphate (dNTPs), 30 pmol each of the primers Xac01 (5'-CGC CAT CCC CAC CAC CAC CAC GAC-3') and Xac02 (5'-AAC CGCT CAA TGC CAT CCA CTT CA-3') (Coletta-Filho *et al*. 2005), 1 µl of bacterial DNA template and 1.25 U of *Taq* DNA polymerase. PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 36 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension at 72°C for 45 s with a single final extension cycle at 72°C for 5 min. PCR products were subjected to electrophoresis through 1.0% (w/v) agarose gels in TBE buffer (0.1 M Tris-HCl, 0.05 M boric acid and 0.01 M EDTA) and stained with ethidium bromide.

Host range and pathogenicity determination

Bacterial strains were cultured on NA medium (Nutrient Agar) and incubated at 28°C. A suspension of approximately $1x10^8$ CFU/ml was prepared from 1-day-old culture (Mohammadi *et al*. 2001). Bacterial suspensions were sprayed on the leaf surfaces of different citrus varieties (Table 2). Inoculated plants were kept under natural light in greenhouse conditions at 25±2°C. The controls were treated with distilled water the same way. Inoculated leaves were covered with freezer bags for 3 days, and symptoms were assessed on young shoots for a month. This experiment was conducted twice.

Analysis of whole-soluble cellular proteins

Sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE) of whole-cell soluble protein strains was carried out in a discontinuous system under denaturing conditions (Laemmli 1970). A bacterial suspension with an optical density of 1 at 600 nm was prepared from each strain after an overnight incubation on NA medium. Each sample was spun down at 5000 g for 5 min. In each sample, 200 µl of sample buffer (65 mM Tris-HCl, 2% Sodium dodecyl sulphate, 1% Mercaptoethanol, 5% Glycerol, Bromophenol blue 0.02%, pH 6.8) was added, boiled for 5 min and centrifuged at 13,000 g for 10 min. Polyacrylamide gel consisted of two parts; stacking gel (5% w/v) and resolving gel (10% w/v). A 50 µl of soluble proteins from each sample was added to slots of stacking gel, and electrophoresis carried out at a constant voltage of 150 V. Afterwards the protein fraction on resolving gel, was stained in dying solution (Coomassie brilliant blue R250, acetic acid, methanol) and destained in the same solution without the dye.

PCR condition of rep-PCR DNA fingerprinting

Genomic DNA extraction was performed by the chloroform-isoamyl alcohol method as described by Hu *et al*. (2007). Four different primers, ERIC-1R, ERIC-2, REP-1R (Versalovic *et al*. 1991) and BOX-A1R (Versalovic *et al*. 1994) were tested for rep-PCR fingerprinting (Table 3). PCR was performed in a final concentration of 25 µl with 2.5 µl of 10 x buffer (CinnaGen, Iran), 30 pmole of each primer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 60 ng of genomic DNA and 2.5 U of *Taq* DNA polymerase. PCR amplification was carried out in a termocycler (Mastercycler® gradient, Eppendorf, Germany), in the following cycles: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C or 48°C for 1 min with REP-1R and three other primers BOX-A1R, ERIC-1R, ERIC-2 respectively, and extension at 72°C for 2 min with the final extension cycle for 10 min. PCR products were resolved in 1.2% agarose gel and TBE buffer at 80 V for 90 min. The gels were stained with ethidium bromide and photographed on a UV transilluminator. DNA

molecular weight markers (GeneRuler™ 1kb and 100 bp DNA ladder, Fermentas) were used to determine the size of amplified fragment.

RAPD-PCR fingerprinting

Thirty-three different 10-mer oligonucleotide primers were tested. The 5 primers 211, 220, 230, 232 and OPA11 (Table 3) were chosen on the basis of their capability to produce polymorphic bands in a preliminary evaluation, and reproducibility for RAPD fingerprinting. Genomic DNA was extracted as described for rep-PCR. The final concentration of $MgCl_2$ and *Taq* DNA polymerase in PCR mixture were 2 mM and 1.5 U, respectively. Concentration of other materials was the same as the rep-PCR condition. PCR amplification was performed in a termocycler (Mastercycler® gradient) in the following cycles: initial denaturation at 94°C for 5 min; 40 cycles of denaturatin at 94°C for 1 min, annealing at 34.5°C with 211 and 220, 35.2°C with 230 and 232 and 36.5°C with OPA11, for 1 min and extension at 72°C for 2 min with the final extension cycle for 10 min.

Data analysis

The results of rep-PCR and RAPD fingerprinting were compared based on the presence or absence of fragments at a specific position (0 absences; 1 presence). The obtained data was calculated with the program NTSYS version 2.1 (Rohlf 2000) based on Jaccard's coefficient and clustered with the unweighted pair group method with arithmetic mean (UPGMA). Marker index of different primers were calculated according to the formula described by Powell *et al*. (1996).

RESULTS

A total of twenty-five *Xcc* strains were isolated from infected Mexican lime from southern Iran. Also one strain of *Xcc* from the Philippines, was isolated from celemantin (*C. reticulata*) and included in this work (Table 1). All strains were Gram negative, obligate aerobic and unable to produce urease. However, they were able to generate hydrogen sulphide from cysteine and hydrolyse starch, gelatin, aesculin, casein and Tween 20 and 80. The alkaline reaction was performed on litmus milk by all strains. They utilized dextrin, maltose, D-manose, lactose, D-melibiose, asparatic acid, glycogen, L-prolin, D-manitol, citrate, lactic acid, salicin and D (+) cellubiose. They could not utilize L-arabinose, Xylitol, raffinose and tartaric acid. Based on these phenotypic tests, the strains were identified as putative *Xanthomonas citri* subsp. *citri*. The identity of the strains as *Xcc* was confirmed by subjecting them to PCR amplification. Strains generating a 581 bp fragment in PCR using primer pairs specific for *Xcc,* were selected and used for analysis of genetic variation.

Based on host range determination, all strains were divided into two groups (Table 2). The first group was pathogenic on Mexican lime, sour orange, citrange and citrumelo. The second group induced symptoms only on Mexican lime. Inoculation of isolates on susceptible varieties, resulted in typical symptoms of CBC disease on leaves and shoots after three weeks (Fig. 1a–d). Canker symptom primarily appeared on the lower surface of leaf tissue and later on the upper surface. Lesions gradually joined together and made erumpent callus-like postules with water-soaked margins. All strains were re-isolated from inoculated leaves and re-identified by phenotypic characters.

The SDS-PAGE technique was repeated three times to analyze whole-cellular soluble proteins. The protein profile was similar in all strains and there was no considerable difference among them.

Different fingerprints were generated by the products of rep-PCR. The primers yielded PCR products that ranged from 200 to 3000 bp. BOX-PCR did not differentiate strains, whereas analysis of ERIC-1R-PCR fingerprints (Fig. 2) yielded two main clusters. One cluster included all strains from the Kerman, Hormozgan and Fars provinces with DH strain. The other cluster included all the strains

Table 1. Code, host plant, location and year of isolation of *X. citri* subsp. *citri* strains used in this study

Strain code	Host plant	Location	Year isolated
K1-K9	*C. aurantifolia*	Kerman province	2007
F1-F7	*C. aurantifolia*	Fars province	2007
H1-H5	*C. aurantifolia*	Hormozgan province	2007
S1-S3	*C. aurantifolia*	Sistan-va-Baluchestan province	2007
DH	*C. reticulate*	Philippine	2007

Table 2. Host range determination of Iranian strains of *X. citri* subsp. *citri*

Host plant	Group 1	Group 2
Mexican lime (*Citrus aurantifolia*)	+	+
Sour orange (*C. aurantium*)	+	–
Citrumelo (*Poncirus trifoliata* × *C. paradise*)	+	–
Citrange (*C. sinensis* × *P. trifoliata*)	+	–
Citrumelo (*Poncirus trifoliata* × *C. paradise*)	–	–
Grapefruit (*C. paradisi*)	–	–
Orange (*C. sinensis*)	–	–
Pamello (*C. grandis*)	–	–
Sweet lime (*C. limettioides*)	–	–

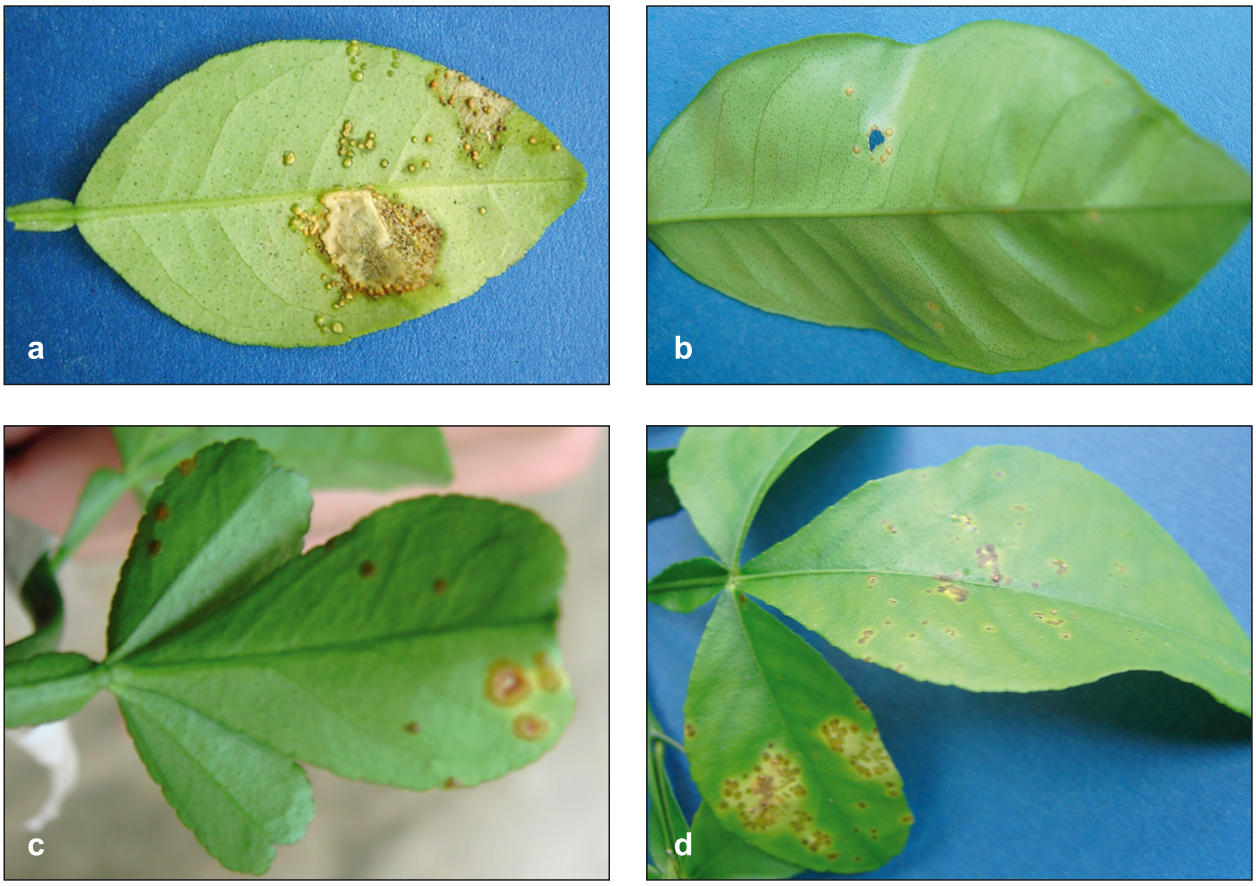


Fig. 1. Symptoms developed by the Iranian strain of *X. citri* subsp. *citri* inoculation on leaves of Mexican lime (*C. aurantifolia*) (a) Sour orange (*C. aurantium*) (b) Citrange (*C. sinensis* x *P. trifoliata*) (c) Citrumelo (*P. trifoliata* x *C. paradisi*) (d)

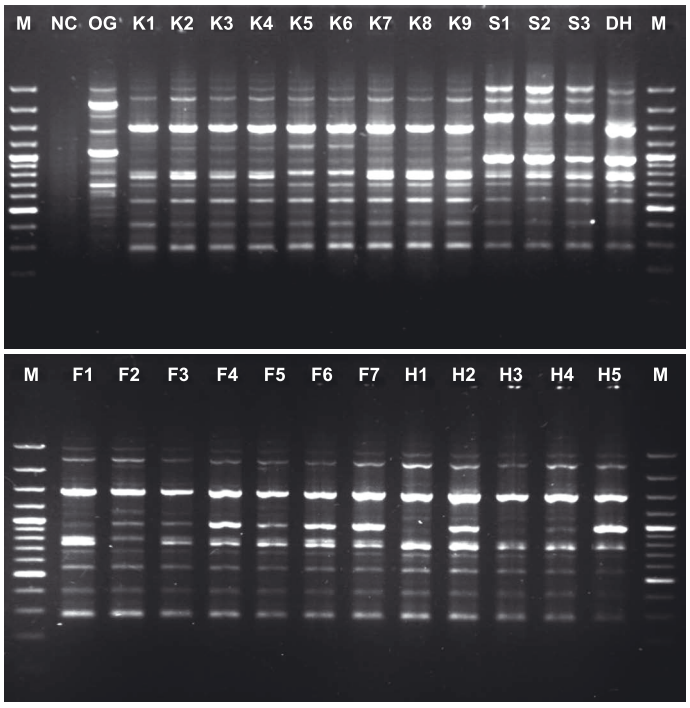


Fig. 2. PCR fingerprinting pattern of genomic DNA of Iranian strains of *X. citri* subsp. *citri* from different geographical regions of Iran generated by ERIC-1R primer. Lane M, molecular marker GeneRuler™ 100 bp DNA ladder (Fermentas); lane NC (negative control) without DNA template; lane OG (out group) *X. citri* subsp. *malvacearum*; lanes K1 to K9, strains from the Kerman provice; lanes S1 to S3, strains from the Sistan-va-Baluchestan province; lane DH, strain from the Philippines; lanes F1 to F7, strains from the Fars province; lane H1 to H5, strains from the Hormozgan province

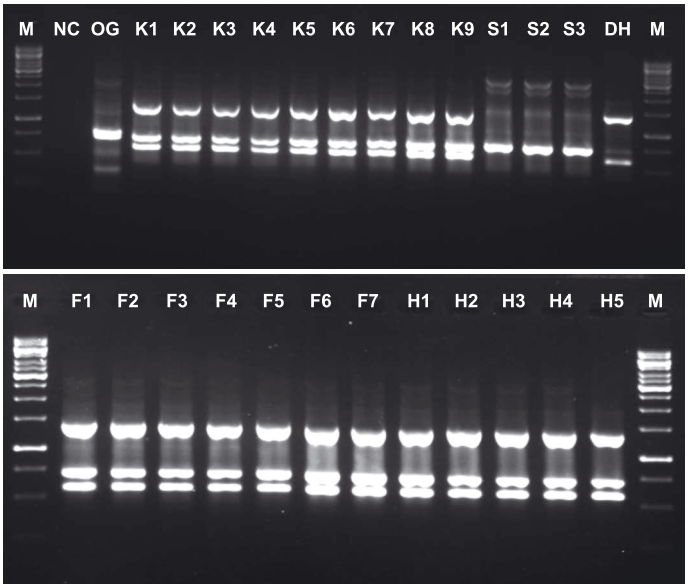


Fig. 3. PCR fingerprinting pattern of genomic DNA of Iranian strains of *X. citri* subsp. *citri* from different geographical regions of Iran generated by primer 232. Lane M, molecular marker GeneRuler™ 1 kb DNA ladder (Fermentas); lane NC (negative control) without DNA template; lane OG (out group) *X. citri* subsp. *malvacearum*; lanes K1 to K9, strains from the Kerman province; lanes S1 to S3, strains from the Sistan-va-Baluchestan province; lane DH, strain from the Philippines; lanes F1 to F7, strains from the Fars province; lane H1 to H5, strains from the Hormozgan province

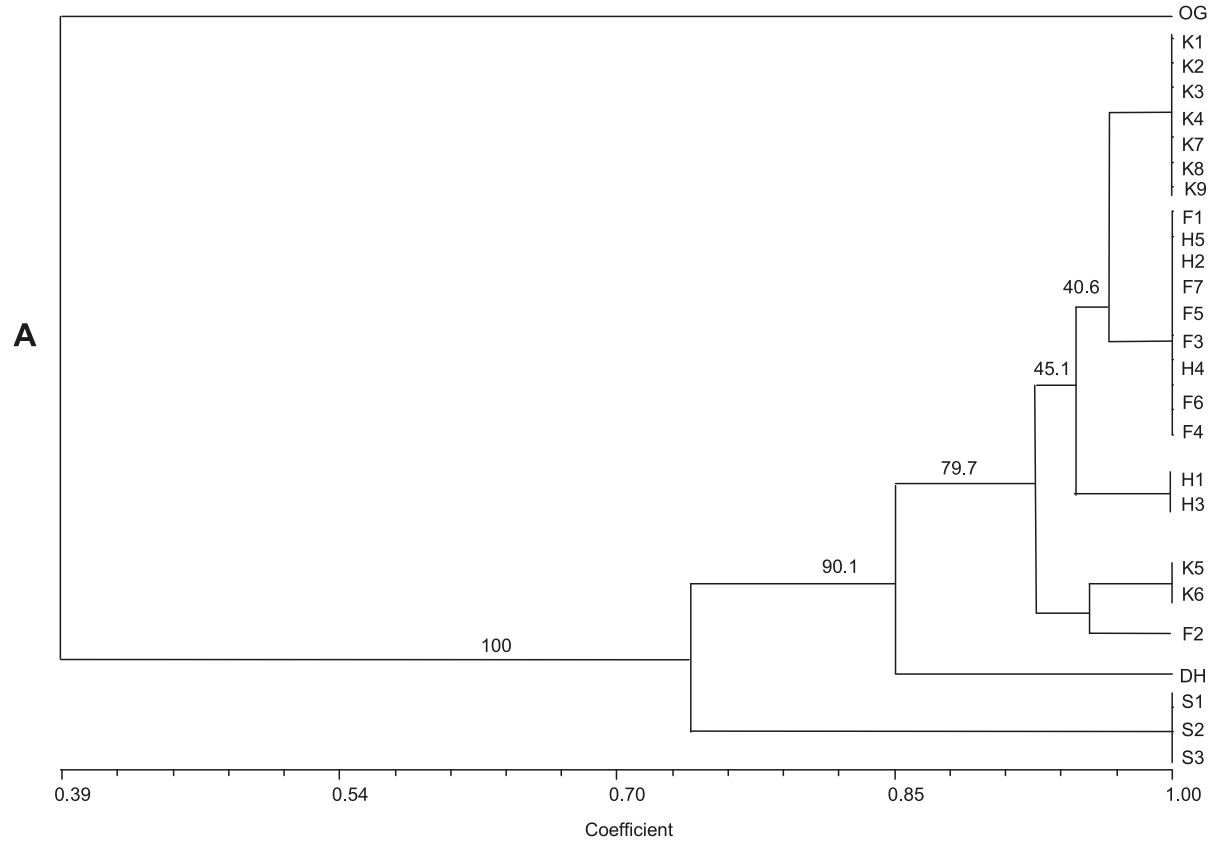
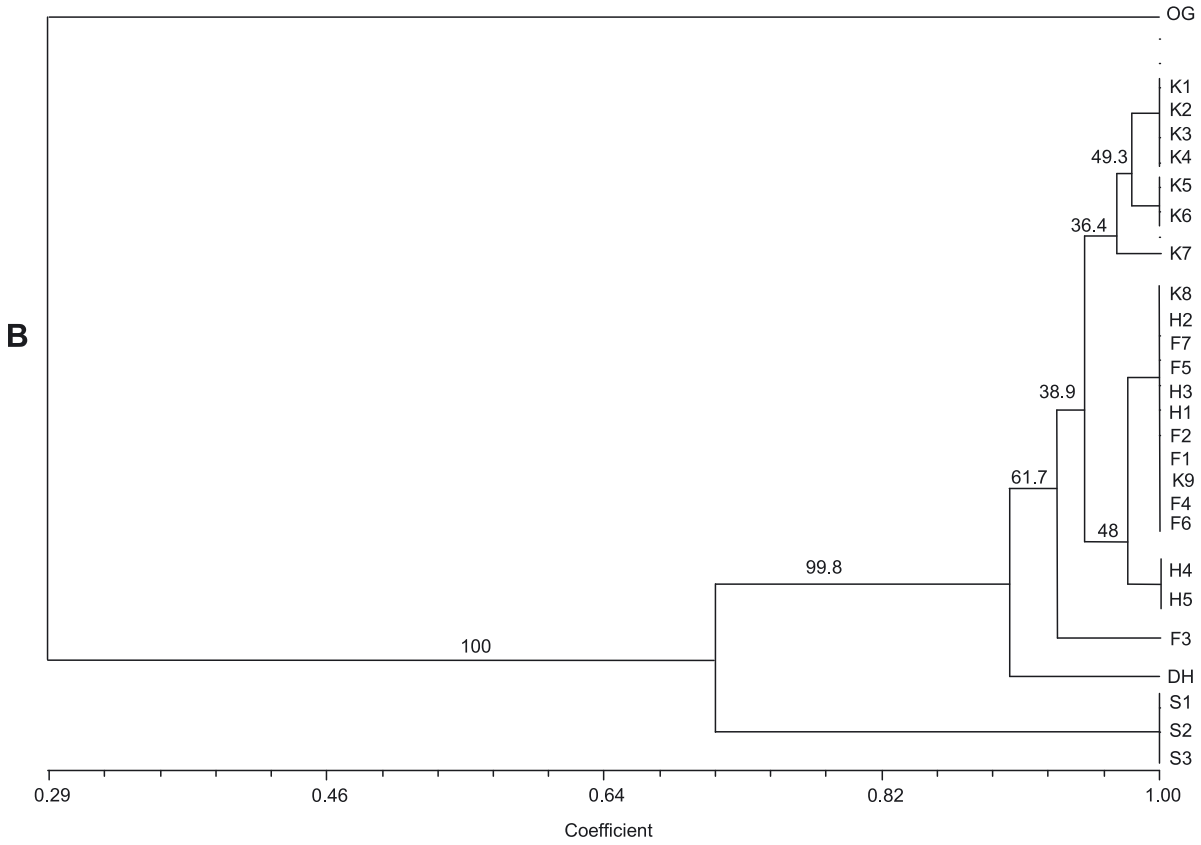


Fig. 4. Dendrogram showing the relationship between 25 *X. citri* subsp. *citri* strains by UPGMA clustering based on rep-PCR (A) and RAPD (B) analysis. Bootstrap values (based on 100 replicates) are indicated at the node. K, F, H, and S stands for strains from the Kerman, Fars, Hormozgan, Sistan-va-Baluchestan provinces, respectively. DH stands for strain from the Philippines



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from Sistan-va-Baluchestan. The mean level of similarity between the two clusters was 53%. In the first cluster, DH strain separated from other strains with about 63% similarity. Strains H1 and H3 and strains K5, K6 and F2 separated as two subgroups from the first cluster with 81 and 88 percent similarity, respectively. The fingerprint clusters by ERIC-2-PCR separated all strains into two main groups with 71% similarity. The first group included all strains except strain from the Sistan-va-Baluchestan province and vice versa. REP-1R-PCR fingerprints yielded two main clusters. One cluster included strains from the Kerman and Sistan-va-Baluchestan provinces, and one strain from the Philippines, and the other cluster included strains from the Fars and Hormozgan provinces. The mean level of similarity between two clusters was approximately 85%. In the first cluster, strains from the Sistan-va-Baluchestan and the Philippines were divided like other strains and had a similarity of 92%. The combination of fingerprints obtained by different rep-PCR primers yielded three main clusters. The first cluster included all strains from the Kerman, Hormozgan and Fars provinces, the second one included the strain from the Philippines, and the third one included all strains from the Sistan-va-Baluchestan provinces at a similarity level of 86% (Fig. 4A).

RAPD marker was used to determine the genetic relationship between Iranian strains of *Xcc*. Primers 211, 220, 230, 232 and OPA11 generated different fingerprints among *Xcc* strains. PCR products of these primers ranged from 100 to 7000 bp long. Based on fingerprint that was generated by primer 211, strains were divided into two main clusters. A total of 3 strains from the Sistan-va-Baluchestan province separated from other strains with a 77% mean level of similarity. In the first cluster, strain F3 as a subgroup separated from the strains of group one with a similarity level of 91%. Analysis of primer 220 yielded two main clusters and strains from the Sistan-va-Baluchestan province, separated from other strains with a similarity level of 71%. In cluster one, two groups could be defined; one that included strains K5, K6 and K7, one that included the strain from the Philippines; with a similarity level of 93 and 81 percent, respectively. Two main clusters that were obtained from fingerprinting of primer 230, separated strains from the Sistan-va-Baluchestan province with a similarity level of 76%. Cluster one was divided into two groups – one that included K1 to K6 and F3 strains, and one that included the other strains with the strain from the Philippines; with a similarity level of 88%. Based on fingerprint generated from primer 232 (Fig. 3), two main clusters were obtained with a similarity level of 37%. In cluster one, strain from the Philippines separated as a subgroup from other strains with a similarity level of 84%. Fingerprint of primer OPA11 just separated strains from Sistan-va-Baluchestan from other strains, with a similarity level of 87%. Based on combined fingerprints of all RAPD primers, all strains divided in three main clusters as described for rep-PCR fingerprint combinations. However these clusters obtained a similarity level of 91% (Fig. 4B).

DISCUSSION

Based on the phenotypic characteristics, all strains were identified as putative *X. citri* subsp. *citri*. Further biochemical properties were consistent with those previously described for pathotype A (Witeside *et al*. 1993; Vauterin *et al*. 1995; Vernière *et al*. 1998; Khodakaramian *et al*. 1999; Mohammadi *et al*. 2001). All strains had the same biochemical properties and it was concluded these tests were not so capable of showing differences between the Iranian strains of *Xcc*. However, there has been evidence of discrimination power in biochemical tests to differentiate these strains from each other in Iran (Khodakaramian *et al*. 1999; Mohammadi *et al*. 2001). The results of such research, though, were not the same, and the discrimination power of biochemical tests was limited to two or three tests. This could be due to a number of reasons, including the different set of strains which were used, or laboratory error. On the other hand, Vernière *et al*. (1998) has reported that phenotypic tests based on carbon source utilization, usually have not discriminated *Xcc*-A*strains from *Xcc*-A. Since specific primers were designed, based on *rpf* region in *Xcc* genome (Coletta-Filho *et al.* 2005), it was predicted that a fragment with 581 bp long was amplified from genome of all the strains. Disease severity of strains within pathotype A on Mexican lime as the main host, could not discriminate these strains from each other (Vernière *et al*. 1998; Buithingoc *et al*. 2009). Grapefruit (*C. paradisi*) is determined as a differential host between narrow-host-range groups (A^*, A^w and C strains) and A group with a wide host range (Bruning and Gabriel 2003). There was no symptom of *Xcc* inoculated strains on grapefruit in this study. The molecular basis for avirulence of narrow-host-range groups on grapefruit is unknown (Al-Saadi *et al*. 2007). Although group one of the strains was pathogenic on four citrus varieties, they were considered as narrow host range strains. The reason for this is because the results showed their host range was limited to acidic citrus, and they were not pathogenic on other citrus varieties. Strains from the Sistan-va-Baluchestan province were pathogenic only on Mexican lime to which the geographical position of this region seems to have played a role. Because this province is so far from other provinces and the most citrus cultivation refers to Mexican lime, it seems that these factors affect the pathogenicity power of the strains. These strains with narrow host ranges represent 'variant clonal subgroups' of *X. citri* subsp. *citri* and the strains described as A^* (Mohammadi *et al*. 2001). AFLP analysis of 57 Iranian *Xcc* strains by Khodakaramian and Swings (2002) yielded two groups, one included fifty strains as A form, with a wide host range, and the other group included seven strains as F form (new form) with a narrow host range. But in the Khodakaramian and Swings study, twenty-five Iranian *Xcc* strains with an identical geographical distribution were determined as narrow host range (A^*) based on host range determination. This may be due to the year of isolation since Khodakaramian and Swings (2002) studied strains isolated in 1996, whereas strains used in this study were isolated in 2007. This difference may also come from Khodakaramian and Swings' host specificity in southern Iran, as their strains were isolated from Mexican lime, orange, grapefruit and sweet lime while our strains were only isolated from Mexican lime. SDS-PAGE analysis of whole cell soluble proteins is not capable of discriminating Iranian *Xcc* strains. By using SDS-PAGE analysis of whole cell soluble proteins, proteins were resolved based on their size, that is why the method is not capable of showing any differences among these strains. However, this method could be used to identify the different CBC species (former pathotype) from each other (Vauterin *et al*. 1991). According to Egel *et al*. (1991) *Xcc*, A strain shared 90% relatedness to *X. citri* subsp. *malvacearum* so we chose this strain as an out group to DNA fingerprinting of strains used in this study. All strains from different provinces in southern Iran except those from Sistan-va-Baluchestan, were separated in different clusters with high similarity. It seems that geographical origin of strains from the Sistan-va-Baluchestan province is different from the geographical origin of other strains. These strains were separated as a different group because there were more fingerprints that were performed by primers of rep-PCR and RAPD markers. The strain from the Philippines (DH) is used in different fingerprints to evaluate which primer(s) could be capable of clustering strains based on their geographical origin. The highest polymorphism among strains was observed by two primers ERIC-1R and 232 with 71 and 75 percentages, respectively (Table 3). Polymorphism information content (PIC) is used to show the genetic distance between different genotypes (Mohammadi and Prasana 2003). Two primers ERIC-2 and 211 with highest PIC value were considered as the best primers to show genetic distance among Iranian *Xcc* strains. Marker index (MI) value is useful to predict the efficiency of a molecular marker for studying on a germplasm (Chadha and Gopalakrishna 2007). Primers ERIC-1R and 232 were determined as two efficient primers causing higher MI value. This is consistent with the results presented here, ERIC and BOX-PCR analysis of worldwide *Xanthomonas* strains causing CBC under specific condition showed discrimination of different pathotypes and subgroups, especially strains A^* and A^w within the pathotype A. Although these strains are closely related to strain A, they were discriminated by rep-PCR analysis (Cubero and Graham 2002). In contrast, Lee *et al.* (2008) were not successful in discriminating strains A^* and A^w as strain A, using the rep-PCR technique. Genetic diversity analysis of *Xcc* strains by the use of RAPD marker has not been reported yet. Primer screening is a necessary step to produce reliable fingerprints (Trebaol *et al*. 2001), so in this research different RAPD primers tested to find the most reproducible. The discriminatory power of rep-PCR is greater than RAPD for differentiating between closely related *Salmonella* isolates (Albufera *et al*. 2009). The difference between percentage polymorphism and the PIC and MI values of two marker primers was not so considerable. It was concluded, that well-chosen primers could result in a quick estimate of genetic diversity, epidemiology and geographical distribution in the studies of *Xcc* strains. However it should be noted, that rep-PCR compared to RAPD is more specific and the results of the rep-PCR marker are more reliable.

Table 3. Primer name and sequence, number of generated bands and degree of polymorphism of amplified DNA in rep-PCR and RAPD analysis of *X. citri* subsp. *citri*

Primer name	Sequence (5'–3')	No. of generated bands	Polymorphism D. [%]
BOX-A1R	CTACGGCAAGGCGACGCTGACG	9	0
ERIC-1R	ATGTAAGCTCCTGGGGATTCAC	21	71.42
ERIC-2	AAGTAAGTGACTGGGGTGAGCG	17	29.41
REP-1R	IIIICGICGICATCIGGC	11	18.18
211	GAAGCGCGAT	14	28.57
220	GTCGATGTCG	17	35.29
230	CGTCGCCCAT	17	35.29
232	CGGTGACATC	8	75
OPA11	TGGACCGGTG	8	12.5

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