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Genomic and pathogenic properties of *Pseudomonas syringae* pv. *syringae* strains isolated from apricot in East Azerbaijan province, Iran

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ABSTRACT

Strains of *Pseudomonas syringae* pv. *syringae* (*Pss*) were isolated from *P. armeniaca* in different geographic areas in East Azerbaijan province, Iran, and studied for genetic diversity and host preference. Results of morphological, physiological and biochemical tests showed no differences among strains and *syrB* gene was determined to be present in all strains by PCR using gene-specific primers. Results of antibiotic assays showed that all strains were resistant to ceftriaxone and erythromycin, while tetracycline induced the strongest growth inhibition. In pathogenicity tests, all strains incited progressive necrotic lesions on apricot twigs at inoculated sites. Severity of symptoms was variable on mango leaves, lemon fruits, bean pods and tomato seedlings. To assess genetic diversity among strains, clustering of strains was performed based on partial sequences of the 16S rRNA and the *rpo*D housekeeping genes and DNA fingerprinting using IS50-PCR analysis. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic (UPGMA) method and Jaccard's similarity coefficients. High genetic diversity was detected among strains. All *Pss* strains were divided into two distinct clusters at the 37% similarity level. In summary, this study indicates that *Pss* strains isolated from apricot in East Azerbaijan province in Iran, are genetically diverse, do not display host specificity, but are heterogeneous in regard to aggressiveness.

1. Introduction

East Azerbaijan province is one of the main hubs for apricot (*P. armeniaca*) cultivation in Iran. Apricot trees are susceptible to a large number of pathogenic agents such as fungal, bacterial, viral and phytoplasmal (Chandel et al., 2011; Gouk, 2016; Šafářová and Navrátil, 2012; Salehi et al., 2015) that cause serious damages to them. One of these pathogens is *Pseudomonas syringae* pv. *syringae*, the causal agent of stone fruit bacterial canker. Stone fruit bacterial canker is one of the most destructive diseases and results in yield reduction of 10–20% in young orchards and even up to 80% under favorable climatic conditions (Kennelly et al., 2007; Young, 1991). Bacterial canker disease caused by *Pss* was reported for the first time on apricot trees in Esfahan province in Iran and resulted in yield reduction of 22–50% (Bahar et al., 1982). Since then, the disease has been detected in most stone fruit orchards in different provinces in Iran (Ahmadi et al., 2017; Al-e-Yasine and Banihashemi, 1993; Karimi-Kurdistani and Harighi, 2008; Khezri et al., 2010; Najafi Pour and Taghavi, 2011). *Pss* belongs to genomospecies I (Young, 1991) based on genomic similarity in DNA–DNA hybridization among *P. syringae* strains and is unique in its ability to cause disease in over 180 species in several unrelated genera including horticultural crops and ornamental and fruit trees (Bradbury, 1986; Gardan et al., 1999; Young et al., 1996). *Pss* strains have shown a high level of similarity in biochemical and serological tests and the common biochemical tests could not separate strains at the intrapathovar level (Cirvilleri et al., 2005; Hildebrand et al., 1982). The large genetic diversity of *Pss* is the likely cause of its wide host range (Martín-Sanz et al., 2013). Studies showed that the lower host range of other pathovars could be a result of the limited diversity within *morsprunorum*, *glycinae* and *tomato* pathovars (Louws et al., 1999). Little et al. (1998) suggested host specificity within the *Pss* strains that cause disease on stone fruits. The *Pss* strains isolated from *Prunus* showed similar genetic profiles using ERIC-PCR analyses, whereas most *Pss* strains isolated from other hosts generated different and variable patterns (Little et al.,

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1998).

Among various molecular techniques used to characterize the *Pss* strains, repetitive elements such as rep-PCR and insertion sequence (IS) 50-PCR, are the common molecular methods for characterization, classification, and diversity studies of bacterial strains between and within *P. syringae* pathovars and bacterial canker disease of stone fruit trees (Bultreys and Kaluzna, 2010; Gilbert et al., 2009; Louws et al., 1994). IS50 belongs to the heterogeneous IS4 family of transposases and use a "cut-and-paste" mechanism. IS50 range in size from 1350 to 1550 bp and consist of a single open reading frame (ORF) (Berg, 1989; Mahillon and Chandler, 1998; Reznikoff, 1993; Siguier et al., 2014). IS50 is a component of the Tn5 transposon. Tn5 is bordered by two IS50 sequences and carries a cluster of antibiotic resistance genes (Mahillon and Chandler, 1998; Tobes and Pareja, 2006). IS50 has been used as a target for primers to investigation the genetic diversity of several bacterial genera including *Pseudomonas*, *Agrobacterium* and *Brenneria* (Falahi et al., 2010; Noble et al., 2006; Rouhrazi and Rahimian, 2012; Weingart and Völksch, 1997).

The 16S rRNA gene is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria but does not provide sufficient resolution to derive clear taxonomic relationships among closely related strains within the same species. Sigma factor 70 (a 70-kDa protein), encoded by *rpo*D, is an appropriate biomarker for phylogenetic and taxonomic analyses of *Pseudomonas* species (Mulet et al., 2010; Yamamoto et al., 2000). The *rpo*D has been shown to be useful in grouping pathovars into phylogroups that largely correspond to genomospecies of *P. syringae* (Bull et al., 2011; Ferrante and Scortichini, 2010; Sănchez et al., 2014).

Considering the importance of apricot as horticultural product in East Azerbaijan province in Iran (Ahmadi et al., 2016) and the damage that *Pss* strains cause on it, the objectives of this study were drafted to investigate the level of genetic diversity among *Pss* strains on apricot trees using the 16S rRNA gene, the *rpo*D housekeeping gene, and IS50-PCR and to determine if there is a relationship between phylogeny and host range.

2. Material and methods

2.1. Bacterial isolation and identification

Bacterial strains were isolated from different cultivars of apricot trees with different cultivation ages in 13 geographic areas (Ajabshir, Azarshahr, Basmenj, Esfahlan, Ilkhchi, Khosro-shahr, Mianeh, Marand, Oskou, Sardroud, Shabestar, Sepidan and Zinjanab) that are the major hubs for apricot production in East Azerbaijan province, Iran, in March-April 2015. Tissues of blossoms and branches from infected trees with symptoms of canker with oozing on woody tissues, blast of blossoms, and spur dieback were selected for isolation.

Five gram of 1 cm crashed healthy and infected tissues were suspended in 20 ml of 0.1 M potassium phosphate buffer and 0.01 M magnesium buffer for 10 and 120 min, respectively on a shaker at 150 rpm. 100 μL of suspension was streaked on nutrient agar (NA) medium (Merck, Germany). After 96 h of incubation at 25 °C, morphologically different bacterial colonies were isolated and classified using Gram staining and fluorescent production on King's B (KB) medium (Biolife, Italy). Purified Gram negative fluorescent colonies were analyzed using catalase reaction, glucose fermentation/oxidation, Levan production, Oxidase reaction, Pectolytic activity on potato slices, Arginine dihydrolase activity and Hypersensitivity Reaction on Tobacco leaves tests (Lelliott et al., 1966; Schaad et al., 2001). Colonies were also analyzed using the GATTa (Lelliott and Stead, 1987) test: Gelatin liquefaction, Aesculin hydrolysis, Tyrosinase activity and Na-Tartrate utilization (Dye, 1969; Fahy and Parsley, 1983; Kersters et al., 1973; Schaad et al., 2001). Strains were stored at −20 °C in 15% (w/v) glycerol.

2.2. Pathogenicity test and host range

Bacterial strains used in pathogenicity and host range tests were grown for 24 h on NA medium at 28 °C and suspended in sterile double distilled water (DDW) at a concentration of 10^7 CFU/ml (OD600 = 0.5). In all tests, sterile DDW was used as a negative control. The pathogenicity test was performed using the same cultivar of one-year-old green apricot twigs. One ml of bacterial suspension was injected into the twigs at three different sites of leaf germination (Little et al., 1998). Inoculated twigs were maintained in high moisture conditions at 28 °C for 14 days. The presence of black necrotic lesions were recorded as positive pathogenic reaction.

Host preference was performed by artificial inoculation of one cultivar of young detached mango leaves, bean pods, immature lemon fruits and tomato seedlings (Aiello et al., 2015; Cazorla et al., 1998; Cirvilleri et al., 2005; Scortichini et al., 2003; Yessad-Carreau et al., 1994). All host plants were inoculated under the same conditions of light, temperature and humidity. Mango leaves were surface-sterilized with 70% ethanol for 1 min, rinsed in sterile water, and inoculated with 20 μl of a bacterial suspension using an insulin syringe needle at several sites around the midrib until the suspension was dispersed along the interveinal tissue. Inoculated leaves were incubated in 85% moist condition at 22 ± 2 °C and disease symptoms were recorded after 10 days. Necrotic areas around the inoculation sites and their progression along the interveinal tissue were considered as positive reaction in pathogenicity. Young bean pods and lemons disinfected in 0.5% sodium hypochlorite for 1 min, rinsed in sterile distilled water, were inoculated by placing 20 μl of a bacterial suspension on the wounded surface using sterile needles at three different sites. After inoculation, bean pods and lemon fruits were incubated in 82% moist condition at 22 ± 2 °C for 10 days. The presence of necrosis, soft-rot, and water soaked lesions was recorded as positive pathogenic reaction. Four-week-old tomato seedlings were also used for host specificity tests. The surface of young leaves was inoculated by placing 20 μl of bacterial suspension containing carborundum (2 g/L) on the leaf. The inoculated plants were maintained in 100% moist condition for 48 h and placed at 22 ± 2 °C for 10 days. Each strain was inoculated on three leaves. The leaves with wilting were recorded as pathogenic reaction. All tests were performed two times with all *Pss* strains in a completely randomized design (CRD).

2.3. Antibiotic resistance

In this assay, five antibiotics with different mode of action were used. Bacterial suspensions with a concentration of 10^7 CFU/ml (OD600 = 0.5) were cultured on NA medium at 28 °C for 48 h. A single colony of each strain was streaked on NA and 6 mm diameter discs for susceptibility test containing Ceftriaxone (30 mcg), Vancomycin (30 mcg), Tetracycline (30 mcg), Erythromycin (15 mcg), and Streptomycin (10 mcg) antibiotics (Padtan-Teb co. Iran) were placed on the medium. Sterile discs without antibiotics were used as a negative control. The Petri dishes were incubated at 28 °C for 24 h. The experiment was performed with 50 treatments each with three replications in a CRD. The inhibition zone around each disc was measured and compared with control.

2.4. DNA preparation

Total DNA was extracted and purified using the CTAB method according to Doyle and Doyle (1990) with some modifications. 24 h-old bacterial colonies were suspended in 800 μL 2% CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 2% CTAB), maintained at 65 °C in a water bath for 60 min. The suspension was deproteinized with 800 μl of chloroform/isoamyl alcohol (24:1 v/v), and centrifuged for 10 min at 13,000 g. 600 μL of supernatant was added to another sterile tube and 400 μl of cold isopropanol was added, maintained in −20 °C for 20 min, and centrifuged for 10 min at

Table 1
Primers' properties and PCR conditions used in this study.

Target gene	Primer name	Primer sequence	PCR conditions	Amplicon size (bp)	Reference
16S rRNA	fD1 rD1	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-AAGGAGGTGATCCAGCC-3′	94 °C 3 min; 35 cycles (94 °C 1 min, 55 °C 1.5 min and 72 °C 2 min); 72 °C 10 min.	1500	Weisburg et al. (1991)
*rpo*D	rpoD-Fp rpoD-Rp	5′-AAGGCGARATCGAAATCGCCAAGCG -3′ 5′-GGAACWKGCGCAGGAAGTCGGCACG -3′	95 °C 5 min; 30 cycles (94 °C 1.5 min, 63 °C 1 min and 72 °C 2 min); 72 °C 10 min.	582	Sarkar et al., 2006
*syr*B	B1 B2	5′-CTTTCCGTGGTCTTGATGAGG-3′ 5′-TCGATTTTGCCGTGATGAGTC-3′	94 °C 4 min; 35 cycles (94 °C 1.5 min, 60 °C 1.5 min and 72 °C 3 min); 72 °C 10 min.	752	Sorensen et al. (1998)
–	IS50	5′-CAGGACGCTACTTGTGT-3′	93 °C 3 min; 35 cycles (94 °C 1 min, 38 °C 1 min and 72 °C 3.5 min); 72 °C 15 min.	variable	Gilbert et al. (2009)

13,000 g. The precipitate was washed in 100 µl of 70% ethanol and centrifuged for 5 min at 5000 g three times. Precipitated DNA was dried at room temperature and was suspended in 50 µl of DDW. Concentration and quality of DNA were determined using a NanoDrop Nano-200 (Allsheng, China) and gel electrophoresis on agarose.

2.5. Identification of Pss strains using PCR assays

16S rRNA oligonucleotide primers fD1/rD1 and a primer pair for the housekeeping *rpo*D gene, rpoD-Fp/rpoD-Rp were used to amplify approximately 1500 and 582 bp-long fragments encoding an internal region of the 16S rRNA gene and the RNA polymerase sigma 70 factor gene, respectively. Two 21-mer specific oligonucleotides B1 and B2 were used for amplification of a 752 bp fragment of the *syrB* gene. All primers were provided by Macrogen Inc. (South Korea) and their properties are listed in Table 1.

PCR was performed in a final volume of 25 µL containing 12.5 µL of 2× Master Mix Red (Ampliqon, Denmark), 2.5 mM $MgCl_2$, 10 pmol of each primer for the respective gene, 8.4 µL H_2O, and 2 µL of template DNA. The reaction was performed in a thermocycler (peQlab Biotechnologie GmbH, Model nano-200, Erlangen. Germany). Five µl of PCR products were run on a 1–1.5% agarose gel in 1 × TBE buffer (100 mM Tris, 500 mM boric acid and 1 mM EDTA) at room temperature and 100 V for 40–60 min. The DNA fragments were stained with FluoroDye DNA Fluorescent Loading Dye (SMOBio, Korea) and photographed under UV light in a gel doc device. The PCR reactions were carried out as Table 1.

2.6. IS50-PCR analysis

IS50-PCR analysis was conducted for all *Pss* strains as well as for a *P. s.* pv. *morsprunorum* 19b strain as a outgroup. The 17-mer IS50 oligonucleotide primer (Macrogen Inc. South Korea) (Table 1) was used to generate fingerprinting profile according to Gilbert et al. (2009). The PCR reaction was performed in a final volume of 25 µl containing 12.5 µL of 2× Master Mix Red (Ampliqon, Denmark), 50 pmol of primer, 8.4 µL H_2O, and 2 µL of template DNA. PCR amplification was performed in a thermocycler model (peQlab Biotechnologie GmbH, Model nano-200, Erlangen. Germany). The PCR reaction was carried out as Table 1. PCR products were separated by electrophoresis on a 3% agarose gel in 1 × TBE buffer at 80 V for 3 h. The DNA fragments were stained with FluoroDye DNA Fluorescent Loading Dye (SMOBio, Korea) and photographed under UV light in a gel doc device.

2.7. Sequence alignment and phylogenicity test

PCR products of the 16S rRNA and *rpo*D genes were sequenced by Macrogen Inc. (South Korea) in both directions. The quality of raw sequencing reads was checked using the FinchTV v.1.4.0 DNA sequence analysis program. Raw sequencing data were edited and trimmed using the SeqMan™ II expert sequence analysis (DNASTAR Inc., Madison, Wis.) program. Obtained sequences were blasted in NCBI (www://ncbi.nlm.nih.gov) using the BLASTN (megablast) tool. Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in the Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura et al., 2013) software.

Phylogenetic trees were constructed using Mesquite v.2.75 software (Maddison and Maddison, 2011). Phylogenic analysis was performed using Bayesian Inference (BI). A Markov Chain Monte Carlo (MCMC) algorithm was used to generate Bayesian phylogenetic trees (Ronquist et al., 2012). BI phylogenetic trees were constructed by Bayesian probabilities and performed using MrBayes v.3.2.2, with 100,000,000 generations, average standard deviation of split frequencies of 0.01, sampling every 1000 generations and setting of the heating parameter to 0.15. After discarding the first 25% of the generations as “burn-in”, the posterior probabilities (Rannala and Yang, 1996) were calculated from the remaining trees. The best model of nucleotide substitution was selected independently for each locus under the Akaike Information Criterion (AIC) (Akaike, 1974) implemented in MrModeltest v.2.3 (Nylander, 2004). Trees were visualized using the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree). Sequence distances displaying percent identity values of each sequence pair were performed using DNASTAR's MegAlign.

Sequences of 15 *P. syrinage* strains were selected as reference strains for DNA sequencing and phylogenic analysis using 16S rRNA and *rpo*D biomarkers. All new sequences of 16S rRNA and rpoD genes generated during this study were deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm. nih.gov) and the accession numbers of the sequences used for the phylogenetic analyses are listed in Supplementary Table 1.

2.8. Data analysis

The amplified fragments of each strain were scored based on comparison with a DNA standard (100 bp Plus 3 kb, Ampliqon). The presence and the absence of a band for each strain was scored as 1 and 0, respectively. The Jaccard's (Sneath and Sokal, 1973), Dice's and Simple Match's (SM) similarity coefficients were used for calculating a distance matrix between strains and a dendrogram was constructed by the unweighted pair-group method with averages (UPGMA) method using the NTSYSpc program version 2.0.1.5 (Exeter Software, Setauket, N.Y.) (Rademaker et al., 1998). A cophenetic value matrix was calculated by using the COPH option and compared with the original similarity matrix by using the MXCOMP option to test the goodness of fit of the cluster analysis (Fariis, 1969).

3. Results

3.1. Strain collection and identification

Among 103 Gram negative purified strains, five aerobic and catalase positive strains with cream colonies on NA and fluorescence-producing on KB medium were identified as *P. syringae* (*Ps*) LOPAT group Ia (+,-,-,-,+) and *syringae* pathovar (*Pss*) based on GATTa (+,+,-,-) tests (Lelliott and Stead, 1987; Lelliott et al., 1966).

Table 2

Phenotypic and pathogenic characterization of *Pseudomonas syringae* pv. *syringae* strains used in this study.

Test/strain	Pss-26	Pss-82	Pss-170	Pss-174	Pss-176
Area of isolation	Ajabshir	Marand	Sepidan	Sepidan	Sepidan
Sample	Br	Br	Br	Br	Br
Gram staining	–	–	–	–	–
Color on NA	cream	cream	cream	cream	cream
Fluorescent in King's B	+	+	+	+	+
Levan	+	+	+	+	+
Oxidase	–	–	–	–	–
Pot rot	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–
HR	+	+	+	+	+
Catalase	+	+	+	+	+
O/F	O	O	O	O	O
Gelatin liquefaction	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+
Tyrosinase activity	–	–	–	–	–
Tartrate utilization	–	–	–	–	–
Pathogenicity on:					
Apricot twig	LN	LN	LN	LN	LN
Bean pod	Ws	LN	SN	Ws	Ws
Lemon fruit	–	NR	R	–	–
Mango leaf	N	N	N	N	N
Tomato seedling	Wi	Wi	Wi	Wi	Wi

Pss: *Pseudomonas syringae* pv. *syringae*; H: Host of isolation; Ap: Apricot; Bl: Blossom; Br: Branch; (+): Positive; (−): Negative; HR; Hypersensitive reaction; O/F: Oxidative fermentative test; Ws: Water-soaked lesion; N: Necrotic lesion; Wi; Wilting; SN: Small Necrotic lesion; LN: Large Necrotic lesion; NR; Necrotic and Rotting lesion; R: Rotting lesion.

Strains producing necrosis on tobacco leaves were considered as positive reaction in the HR test. These strains were collected from orchards in three geographic areas including Ajabshir (Pss-26), Marand (Pss-82), Sepidan (Pss-170, Pss-174, and Pss-176). All strains were isolated from branches of apricot trees (Table 2).

3.2. Pathogenicity and host range tests

In pathogenicity test, all strains showed progressive necrotic lesions on apricot twigs. All the Pss-26, Pss-82, Pss-170, Pss-174, and Pss-176 strains were found to be of high virulence producing deep and wide necrotic lesions (in a size range of 4–6 mm length and 8–10 mm width) at the injection sites (Table 2 and Fig. 1-1). In the host range assay, each strain induced different symptoms. On mango leaves, all strains were of similar virulence and induced a limited necrotic lesion (1–2 mm diameter) at the sites of injection without progressing along the interveinal tissue (Table 2 and Fig. 1-2). Also the tomato seedlings had the same reaction to all strains. All strains induced sever leaves wilting compared to the control (Table 2 and Fig. 1-3). On bean pods, the Pss-82 strain was the most virulent producing intensive large dark brown necrotic lesions (5 mm diameter) around the injection sites. Other strains, as the Pss-170 strain produced small dark brown lesions (2 mm diameter) around the injection sites and the Pss-26, Pss-174, and Pss-176 strains only induced water soaked lesions around injection sites (2 mm diameter) and were thus considered less virulent (Table 2 and Figs. 1-4). On lemon, the Pss-82 strain was the most virulent and produced progressive necrosis and soft rot lesions around injection sites (in a size range of 3–6 mm diameter). The Pss-170 strain was less virulent producing only soft rot lesions around the injection sites (in a size range of 1–3 mm). The Pss-26, Pss-174, and Pss-176 strains instead produced no symptoms (Table 2 and Fig. 1-5). Koch's postulates were performed 10 days after inoculation and large populations of the same type of bacterial colony were re-isolated on KB medium from infected necrotic tissues after 48 h. The re-isolated strains were identified as *Pss* using biochemical tests and the B1/B2 primers using PCR.

3.3. Resistance to antibiotics

The *in vitro* assay results showed that studied *Pss* strains had large variable levels of resistance to different antibiotics. All five *Pss* strains were resistant to ceftriaxone (30 mcg) and erythromycin (15 mcg) antibiotics (Fig. 2). The Pss-170 and Pss-176 (isolated in Sepidan) strains were resistant to all five antibiotics and the Pss-26 strain (isolated in Ajabshir) was the most sensitive strain to tetracycline, streptomycin, and vancomycin (Fig. 3). Tetracycline (30 mcg) induced the strongest inhibition on bacterial strains isolated from different geographic area by producing an average of 13.9 mm of inhibition zone. However, Streptomycin at low concentration (10 mcg) had a higher inhibitory effect (average, 11.7 mm) than vancomycin at 30 mcg (average, 4.2 mm).

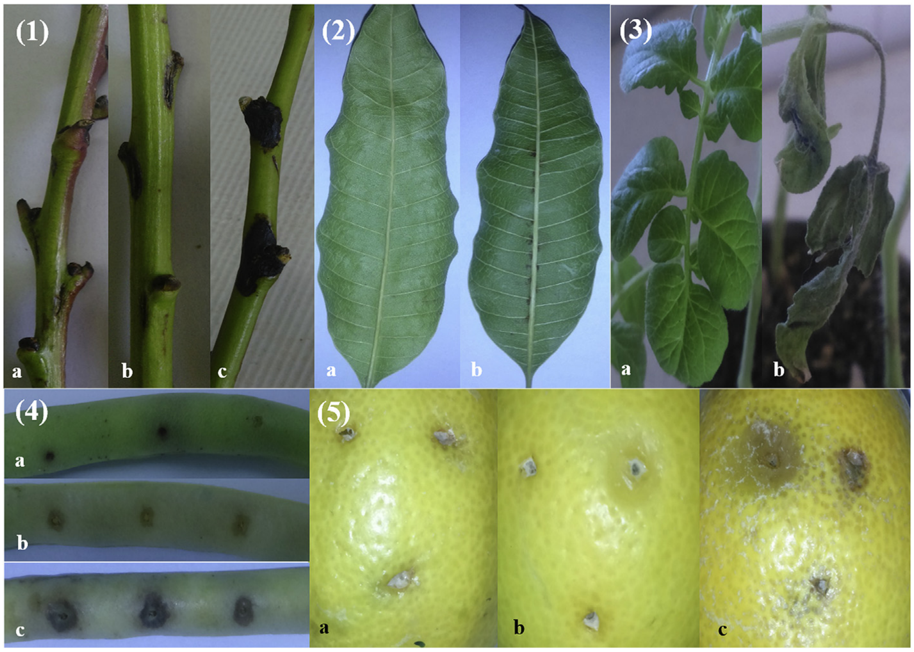

Fig. 1. Symptoms induced by inoculation with *Pseudomonas syringae* pv. *syringae* strains on apricot twigs (1), mango leaves (2), leaves of tomato seedlings (3), bean pods (4), and lemon fruits (5) in pathogenicity tests. (1): necrotic lesions on apricot twigs at site of inoculations caused by less (b) and high (c) virulent strains compared with negative control (a). (2): necrotic lesions on mango leaves at site of inoculations (b) compared with negative control (a). (3): tomato leaves wilting (b) compared with negative control (a). (4): water soaked (b) and necrotic (c) lesions on bean pods compared with negative control (a). (5): rot and necrotic lesions on lemon fruits caused by less (b) and high (c) virulent strains compared with negative control (a).

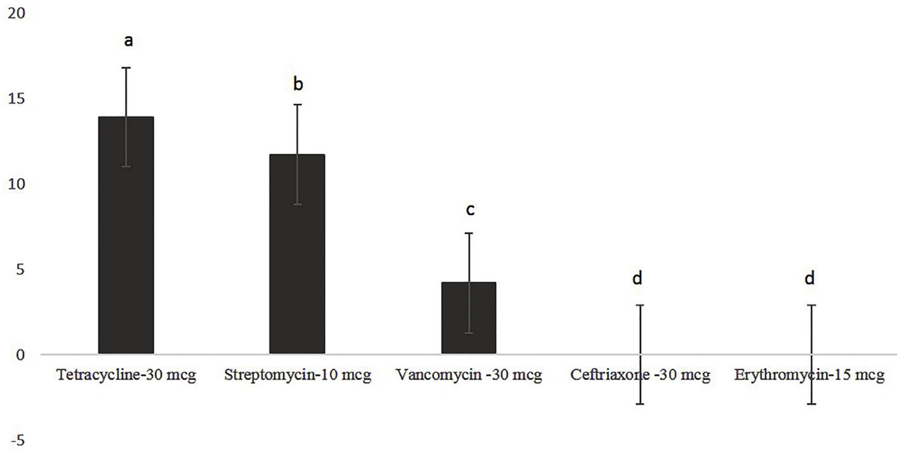

Fig. 2. Average comparison of inhibition effects of different antibiotics on five *Pseudomonas syringae* pv. *syringae* strains. Different letters indicate significant differences between means using Duncan's Multiple Range Test ($p < 0.01$). Data are the means of three replications.

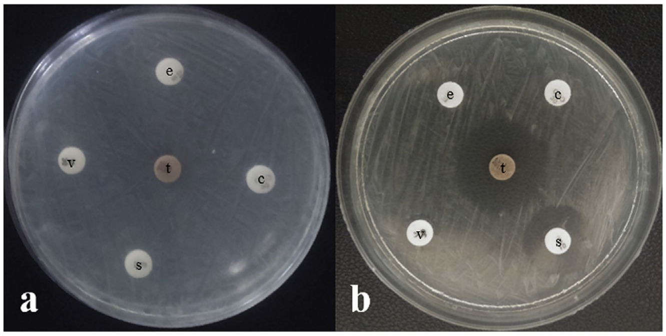

Fig. 3. Different reactions of two *Pseudomonas syringae* pv. *syringae* strains (a: Pss-170 and b: Pss-26 strains) to vancomycin (v), tetracycline (t), erythromycin (e), streptomycin (s), and ceftriaxone (c).

3.4. PCR assays

Using fD1/rD1 and rpoD-Fp/rpoD-Rp pair primers, fragments of ~1500 bp, ~532 bp were amplified from all five strains. The presence of the *syr*B gene, required for syringomycin production in *Pss*, was also tested using B1/B2 primers and all strains amplified the expected 752 bp-long fragment (Fig. 4).

3.5. DNA sequencing and phylogenetic analysis

Amplification of the two loci (16S rRNA and *rpo*D genes) in five *Pss* strains isolated from *P. armeniaca* resulted in fragments of average sequence length of approximately 1132 and 551 bp, respectively. Edited 16S rRNA and *rpo*D gene sequences were aligned to the sequences deposited in NCBI's GenBank database. Based on 16S rRNA gene sequence analysis, Iranian *Pss* strains exhibited greater than 99% similarity (pairwise nucleotide identity) to different sequences of *Pss* strains deposited in NCBI's GenBank database (Supplementary Table 2). In the *rpo*D gene sequence analysis, Iranian *Pss* strains exhibited greater than 96% pairwise nucleotide identity (sequence distances) to sequences of reference *Pss* strains in NCBI's GenBank database (Supplementary Table 3). The best-fit statistical model based on results from MrModeltest was determined for each locus and technique. Hasegawa-Kishino-Yano and proportion of Invariant sites with Gamma distribution (HKY + I + G) was recommended for the 16S rRNA gene fragment. General Time Reversible with Gamma distribution (GTR + G) was recommended for the *rpo*D. General Time Reversible with Gamma distribution and proportion of Invariant sites (GTR + G + I) was recommended for the combined two loci.

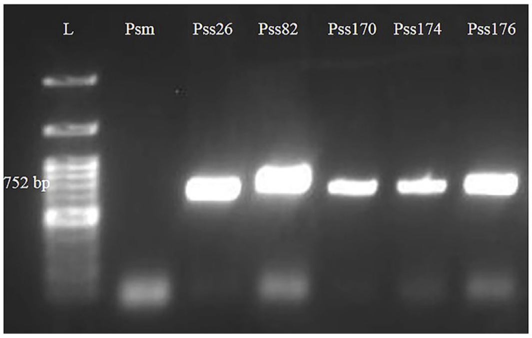

Fig. 4. Agarose gel 1% electrophoresis of PCR products of *Pseudomonas* str ains using primers B1/B2. L: 100 bp DNA ladder; *Pss*: *Pseudomonas syringae* pv. *syringae*; *Psm*: *P. syringae* pv. *morspronorum* 19b.

All trees were supported by high posterior probability values. Phylogenetic trees using Bayesian inference were constructed to evaluate the relationships among the five representative *Pss* strains and 15 reference strains based on individual sequences of the 16S rRNA (798 nucleotides) (Supplementary Fig. 1) and the *rpo*D (536 nucleotides) genes (Supplementary Fig. 2) and the concatenated sequences of both loci (1223 nucleotides) (Fig. 5). In the constructed dendrogram based on the two concatenated sequences, all collected *Pss* strains clustered into two main groups with high posterior probability.. Four strains clustered in the main group I. The main group I was separated into two subgroups in which subgroup I included the Pss-170, Pss-174, and Pss-176 strains and subgroup II the Pss-26 strain. The main group II only included Pss-82 strain isolated in Marand. Relationship between geographic location of strains and phylogeny has been detected. So that, strains from Ajabshir, Marand, and Sardruod were clustered in different subgroups.

3.6. IS50-PCR analysis

DNA fingerprints of five *Pss* strains were generated and analyzed using IS50-PCR (Fig. 6). All strains showed different banding patterns. The number of amplicons in each *Pss* strain varied and ranged from 9 to 13 fragments. The maximum number of amplicons was found in strain Pss-82 isolated from apricot in Marand. The length of fragments ranged from 200 bp to over 3000 bp. The Pss-170 and Pss-174 strains had the smallest fragment and the Pss-26 strain had the largest one. Up to 84.2% of fragments were polymorphic based on IS50-PCR.

A dendrogram was constructed using Jaccard's similarity coefficients with UPGMA for IS50-PCR results. A cophenetic correlation coefficient value of 0.95 indicated a very good fit of the clustering with

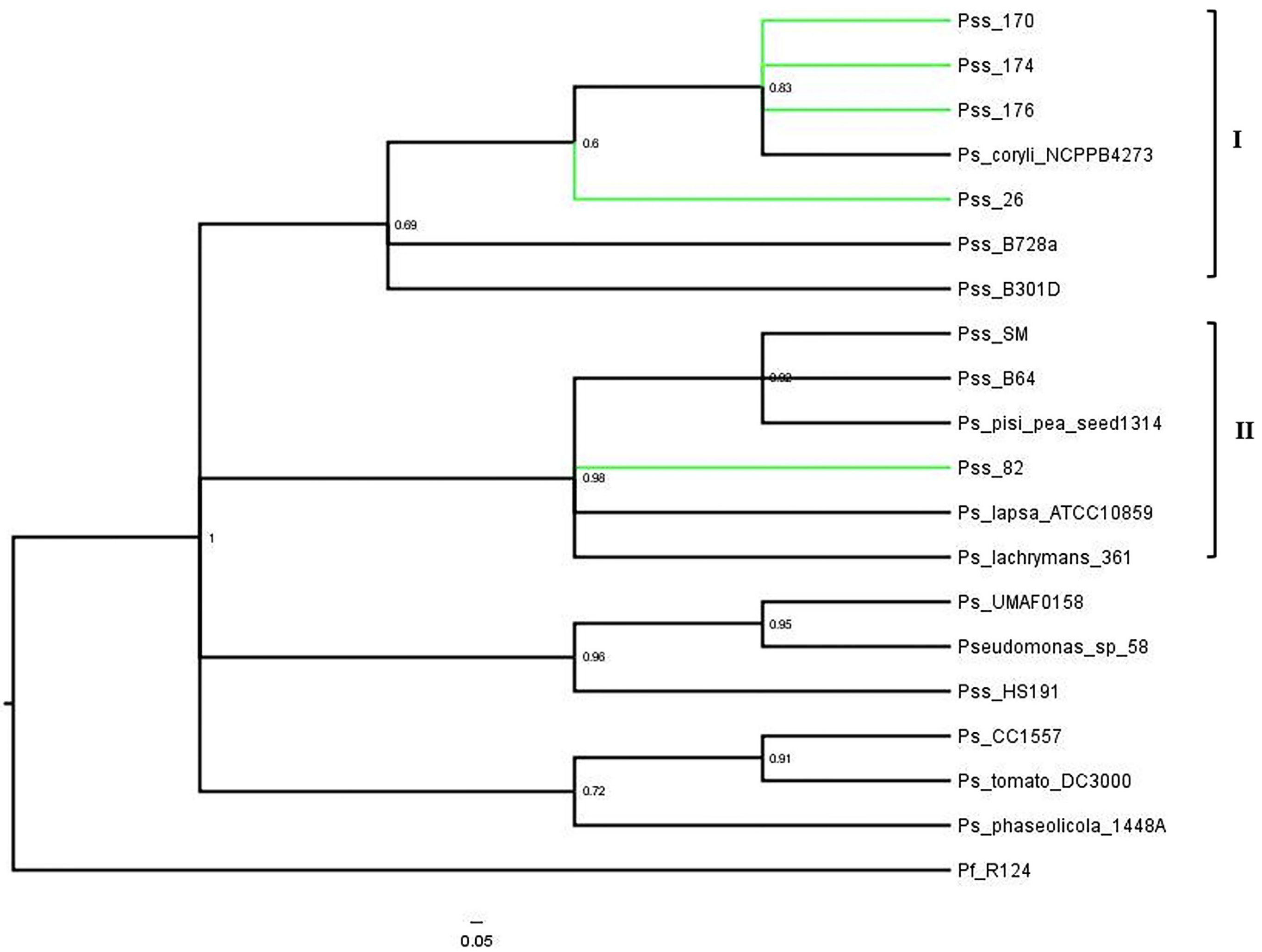

Fig. 5. Bayesian inference phylogenetic tree was constructed for 20 strains of the genus *Pseudomonas*based on aligned concatenated sequences of the 16S rRNA and the *rpo*D genes (1223 nucleotides) using GTR + G + I model. The scale bar represents the average number of substitutions per site, and posterior probability values are shown at the nodes obtained for 100,000,000 generations. Bold black lines in right show the Iranian strains' grouping. *Pss*: *Pseudomonas syringae* pv. *syringae*; Pf: *Pseudomonas fluorescens;* Ps: *Pseudomonas syringae*; green branches show *Pseudomonas syringae* pv. *syringae* strains isolated in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the original similarity matrix. The other dendrograms (data not shown) obtained from Dice and SM had cophenetic correlation coefficients of 0.92 and 0.91, respectively. The resulting dendrogram based on Jaccard's similarity coefficients (Fig. 7) suggests high diversity among the genomic fingerprints of the five analyzed *Pss* strains from apricot, which formed two main groups in the dendrogram at the 37% similarity level and were separated from the outgroup (*Psm*). Main group I included only the Pss-26 strain isolated from apricot in Ajabshir. Main group II divided into two subgroups, included the Pss-82 and Pss-176 strains in subgroup I isolated in Marand and Sepidan, respectively that was divided at a 58% similarity level. Subgroup II included the Pss-170 and Pss-174 strains isolated in Sepidan showed high similarity level of 91%. These two subgroups were divided at a 43% similarity level.

4. Discussion

Strains of *P. syringae* pv. *syringae* have the ability to infect a large variety of woody and herbaceous plant species and are characterized by a high degree of genetic variability. In the present study, we evaluated the genetic, pathogenic, and phylogenetic diversity in a collection of *Pss* strains isolated from apricot trees in East Azerbaijan province in Iran. In the present study, analyzing isolates from 13 geographic areas with severe canker disease symptoms on apricot trees, only ~5% of collected strains were identified as *Pss* strains. However, the low percentage of pathogenic *Pss* strains, found in the current study suggests that additional biotic and abiotic factors besides *Pss* contribute to canker disease in East Azerbaijan province and demands further studies. On the basis of biochemical and physiological tests, all collected *Pss* strains showed no differences in these properties.

The collected *Pss* strains were found to be of the same virulence on the same cultivar of apricot twigs. Our results are in agreement with Abbasi et al. (2013) who reported that *Pss* strains isolated from stone fruits in Iran had the same virulence level on wound-inoculated peach seedling stems and induced cankers of similar severity.

In host preference assays, just one cultivar for each host was used. Yan et al. (2008) suggested that using only one cultivar per plant hosts gives a sufficient approximation of host range. Different levels of virulence were observed among *Pss* strains when inoculated on lemon fruits, bean pods, and no differences were detected on mango leaves and tomato seedlings. Different levels of symptom severity of *Pss* strains on hosts such as lemon fruits, nectarine fruits, tomato seedlings, mango leaves, and bean pods had been previously reported (Cirvilleri et al., 2005; Gašić et al., 2012). For other pathovars instead, more restricted host ranges have been found. For example, *P. s.* pv. *coryli* (Scortichini et al., 2005) was reported to incite severe twig diebacks only on hazelnut. In our study, all *Pss* strains had the same virulence on mango

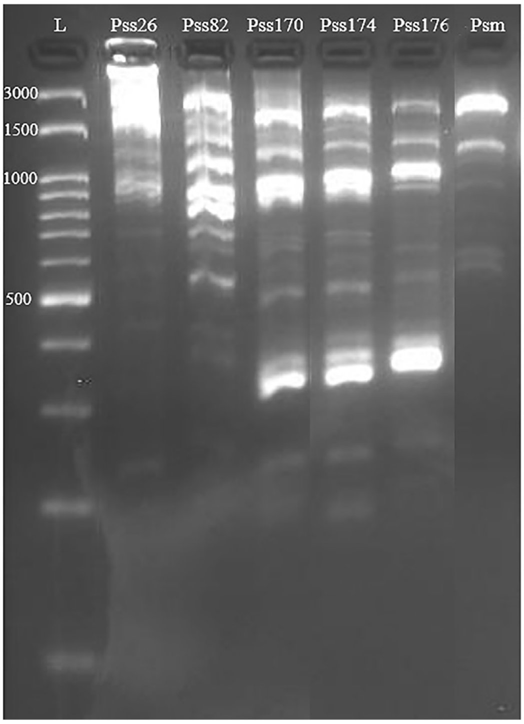

Fig. 6. Genomic fingerprints of *Pseudomonas syringae* pv. *syringae* isolates generated by IS50 primer in 3% gel agarose. L: 100 bp DNA ladder; *Pss*: *Pseudomonas syringae* pv. *syringae; Psm*: *P. syringae* pv. *morspronorum* 19b.

and tomato hosts. However, on lemon fruits and bean pods, the Pss-82 strain, isolated from Marand, induced the most severe symptoms compared to the other strains. Even strains isolated from the same area (Pss-170, Pss-174, and Pss-176 strains isolated in Sepidan) showed different virulence on lemon fruits and bean pod. This result suggests that these hosts can be an alternative host for *Pss* strains isolated from apricot. Bultreys and Kaluzna (2010) declared that pathogenicity tests on different hosts can improve our understanding of the pathogenicity of *P. syringae* strains and provide tools for the better identification and characterization of isolates. Sarkar et al. (2006) suggested that there are multiple ways in which *P. syringae* isolates can adapt to the same host. Such studies can provide insights into the evolutionary mechanisms underlying host adaptation.

Five antibiotics with different mode of action were used in the present study. (i) streptomycin, which inhibits protein synthesis by inactivating the 30S ribosome; (ii) tetracycline, which inhibits chain elongation during protein synthesis by blocking aminoacyl tRNA binding at the A site; (iii) erythromycin, which prevents protein synthesis by binding to the tRNA binding site on the 50S subunit causing the tRNA molecules to dissociate from the ribosomes, (iv) vancomycin, which is a glycopeptide antibiotic and inhibits cell wall synthesis, and (v) ceftriaxone, which is a β-lactam family of antibiotics that inhibits bacterial cell wall synthesis by binding to transpeptidases (Davies and Wright, 1997; Lemke and Williams, 2013.; ; McDermott et al., 2003) Based on antibiotic resistant tests, *Pss* strains showed different susceptibility. These strains were resistant to ceftriaxone and erythromycin and sensitive to vancomycin, tetracycline and streptomycin antibiotics. These results are in agreement with Mohammadi et al. (2001) and Khodayegan et al. (2012) that reported sensitivity of *Pss* strains, the causal agents of canker disease of stone fruit trees in different geographical area in Iran to tetracycline and streptomycin antibiotics. There was no correlation between antibiotic resistance in strains and their virulence level on hosts and area of isolation. Strains collected from Ajabshir (less virulent strain) and Marand (highly virulent strain) were the most resistant strains to antibiotics. Strains isolated from the same geographic area such as Sepidan showed different resistant levels to antibiotics as well. These results indicate the presence of different types of antibiotic resistance elements that determine the strains' resistance and can be used for detecting and grouping *Pss* strains.

Because the production of lipodepsinonapeptide toxins is considered an important virulence factor in *Pss* strains, amplification of the *syrB* gene encoding syringomycin can greatly assist the identification of putative *Pss* strains (Young, 1991). All five *Pss* isolated strains amplified a 752-bp fragment using the B1/B2 primers. Detection of the *syr*B gene in *Pss* strains, as a useful and fast method in identification of *Pss* strains (Sorensen et al., 1998), has been reported in several studies (Gilbert et al., 2009; Kaluzna et al., 2010; Scortichini et al., 2003).

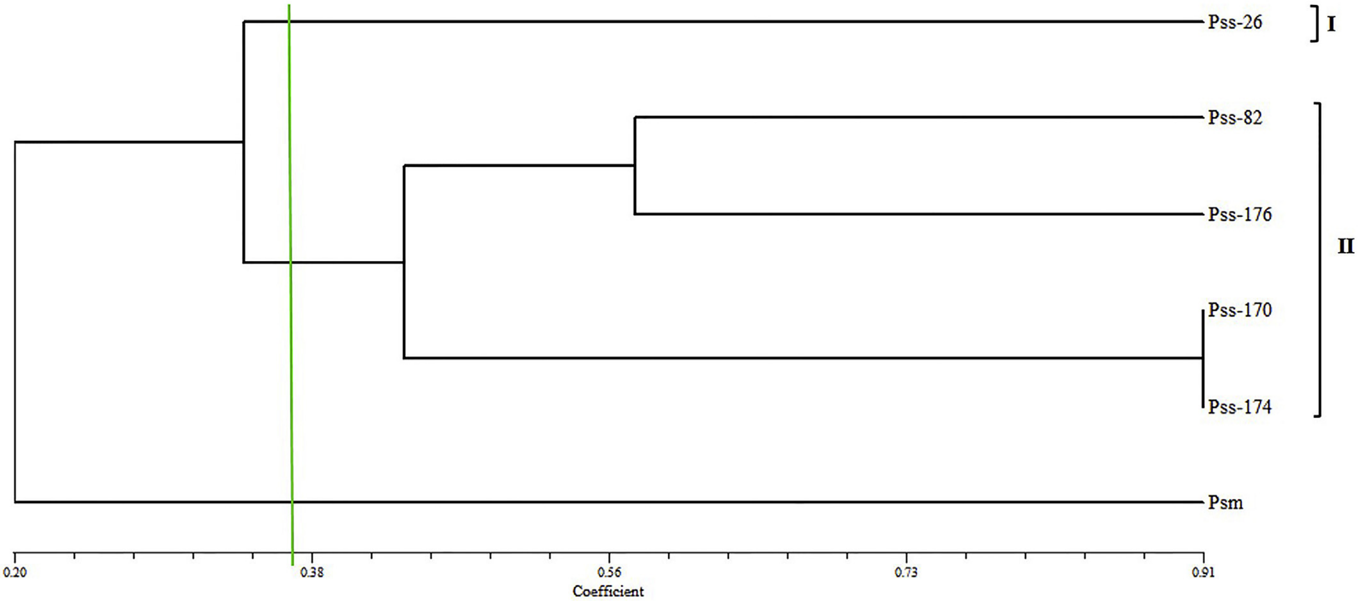

Fig. 7. Dendrogram based on genetic similarity of five *Pseudomonas syringae* pv. *syringae* strains isolated from apricot using IS50 primer. Dendrogram was constructed using UPGMA analysis and Jaccard's coefficient. Bold black lines in right show the Iranian strains' grouping. *Pss*: *Pseudomonas syringae* pv. *syringae*; *Psm*: *P. syringae* pv. *morsprunorum* 19b.

Sequence analysis of the 16S rRNA gene and of housekeeping genes as well as repetitive elements such as IS50-PCR have been described as useful methods for identification and classification and for diversity studies of strains belonging to *P. syringae* (Bultreys and Kaluzna, 2010; Hwang et al., 2005; Louws et al., 1994). Yamamoto et al. (2000) reported that 16S rRNA does not have sufficient taxonomic resolution in differentiation of *Pseudomonas* members even at the intrageneric level. Our results are in agreement with this study. In many studies, the single-copy protein-encoding *rpo*D gene provided more discriminatory information than the 16S rRNA gene (Ghyselinck et al., 2013; Mulet et al., 2009; Parkinson et al., 2011; Rajwar and Sahgal, 2016; Songyu et al., 2017). In our study, the topology of the constructed phylogenic tree based on the *rpo*D gene was different from the 16S rRNA tree and was able to separate strains from different geographic areas.

The concatenated sequences of the 16S rRNA gene fragment and the *rpo*D gene fragment have been used for constructing a dendrogram. In this dendrogram, results showed that there was correlation between host range and clustering of strains. Strains with broad host range and high virulence level, as the Pss-82 clustered in the separate clade from the strains with narrow host range and low virulence level, as Pss-26 strain.

Sawyer et al. (1987) suggested that the high mobility of IS elements could be used to distinguish strains that are very similar or identical based on biochemical assays. Lawrence et al. (1989) supported the theory that since IS fingerprints change so rapidly, only closely related strains can be examined for phylogenetic relationships as well as diversity analysis (Mahillon and Chandler, 1998). In the present study, the five *Pss* strains clustered in two main groups using IS50-PCR. The banding pattern of the DNA fingerprints showed many different amplified bands (84.2% polymorph), indicating that *Pss* strains were different from each other, even strains isolated from the same geographic area. Two main groups in the tree shown in Fig. 7 with high diversity at a 37% similarity level and separated from each other based on geographic area. In main group I included the Pss-26 strain collected in Ajabshir and main group II included the other strains collected both in Marand and in Sepidan. In the constructed tree, no correlation could be found between clustering and host range. In fact, the strains (Pss-26, Pss-170, and Pss-174) with clustering in different groups showed the same virulence level on different hosts and the strain with high virulence level on all hosts, Pss-82, clustered with less virulent strain, Pss-176, in the dendrogram based on IS50.

We conclude that different varieties of apricot grown in different areas of East Azerbaijan province can affect the genetic diversity of *Pss* strains. Our results, despite of limited sample size, are in agreement with the results obtained in other studies that suggested *Pss* strains obtained from various stone fruit trees from different areas in Iran have high genetic diversity based on rep-PCR and IS50-PCR markers (Abbasi et al., 2013; Mosivand et al., 2009; Sedighian et al., 2014). These results are in accordance with Weingart and Völksch (1997), who found that the IS50-PCR fingerprints of the related strains of *P. syringae* pathovars *syringae*, *aptata*, and *atrofaciens* were clearly distinguishable. Also, Sundin et al. (1994) reported a high level of diversity among *P. s.* pv. *syringae* strains isolated from pear nurseries in Oklahoma with IS50-PCR method. Mazzaglia et al. (2011) found the differentiation of *P. syringae* pathovars using IS50-PCR. They results showed the separation of *P. s.* pv. *actinidiae* strains isolated from kiwifruit in Italy from the strains related to pathovars *theae*, *syringae*, and *viridiflova*. Abbasi et al. (2013) reported that compatibility of genetically heterogeneous *Pss* strains on stone fruit trees in Iran along with the long history of cultivating *Prunus* species, has resulted in high diversity among Iranian *Pss* strains. The obtained results are in agreement with Scortichini (2005) who noticed that hosts, environmental factors, and new agronomic techniques all can have a major influence on bacterial population structure.

5. Conclusion

Stone fruit canker disease caused by *Pseudomonas syringae* pv. *syringae* has been detected in most stone fruit orchards in different provinces in Iran. In this study, on the basis of biochemical and physiological tests, all collected *Pss* strains showed no differences in these properties. All *Pss* strains were found to be of the same virulence on apricot twigs. Host range results showed that different levels of virulence observed among *Pss* strains when inoculated on lemon fruits, bean pods, and no differences were detected on mango leaves and tomato seedlings. Insertion sequence 50-PCR, is one of the common molecular methods for characterization, classification, and diversity studies among *P. syringae* pathovars and bacterial canker disease on stone fruit trees. In present study, the Iranian *Pss* strains clustered in two main groups using IS50-PCR. The banding pattern of the DNA fingerprints showed 84.2% polymorphism, indicating that *Pss* strains were different from each other, even strains isolated from the same geographic area. Results of the phylogenic tree based on *rpo*D gene showed that there was correlation between virulence level on hosts and clustering of *Pss* strains. The results obtained from the genomic and host range variability of *Pss* strains isolated in East Azerbaijan province can be used for epidemiological studies and development of management strategies for bacterial canker disease of apricot.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101167.

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