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Exploring Possible Variation among Iranian *Erwinia amylovora* Strains Using Multilocus Typing and Tandem Repeat Analysis

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ABSTRACT

In the current study, molecular typing of 50 *Erwinia amylovora* strains related to different regions in Iran was evaluated using multi-locus sequence analysis and variable number of tandem repeats. In the first assay, phylogenetic tree based on partial sequences of recombinase A, sigma factor S and a heat shock protein GroEL showed significant identity in studied gene sequences. A single nucleotide variation in *gro*EL was determined in IrGh59 strain related to *Crataegus* spp. from Ghazvin Province. In VNTR analysis, the same fingerprinting profile similar to *E. amylovora* reference strain ATCC49946 was yielded for tested strains except NBQ1 and MQ1 which may reflect a unique contaminating source for this disease in Iran. In addition, the honey-bee movements with respect to blossom season probably have a considerable role in fire blight unique dispersal in our area. The NBQ1 and MQ1 strains generated different VNTR profiles, isolated from cultivars Neishabour and Esfehan of *Cydonia oblonga* plant, respectively. No definite assessment can be expressed in this case. However, possible entry of other infection mass from neighboring countries should be determined. Overall, VNTR profile analyses are recommended as a tool to evaluate genetic differences in *E. amylovora* populations. In addition, employing more strains from different known sources could be assistance to achieve more accurate results about *E. amylovora* genetic variation and also fire blight distribution patterns.

Keywords: Fire blight, Housekeeping genes, Iran, VNTR.

INTRODUCTION

The enterobacterium *Erwinia amylovora* is a threat to global pome fruit production and the bacterium can infect a wide host range of plants in Rosaceae family (Palacio-Bielsa *et al.,* 2012). This pathogen has the potential to easily cause considerable damage and become a limiting factor to apple and pear orchards as well as other host plants (Pulawska and Sobiczewski, 2012; Taghdareh *et al.,* 2014). A lack of efficient management strategy, places *E. amylovora* as one of the most important bacterial plant pathogens worldwide (Mansfield *et al.,* 2012).

An obvious discrepancy among strains of *E. amylovora* is its plasmid content (Sebaihia *et al.,* 2010; Powney *et al.,* 2011) that influences the pan-genome of this homogenous species and causes difference in virulence and host range (Llop *et al.,* 2012). Different molecular typing methods are applied for *E. amylovora* differentiation (Pulawska and Sobiczewski, 2012). Most molecular techniques such as random amplified polymorphic DNA (RAPD), ribotyping and Pulse Field Gel Electrophoresis (PFGE) following digestion

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of genomic DNA, can distinguish between strains from *Rubus* and Spiraeoideae host plants (Momol *et al.,* 1997; McManus and Jones, 1995; Jock *et al.,* 2002), while often failing to distinguish strains within the Spiraeoideae group. Although, most molecular methods often fail to distinguish *E. amylovora* strains from Spiraeoideae (Gehring and Geider, 2012; Bühlmann *et al.,* 2013), simultaneous application of two or three methods can be recommended (Donat *et al.,* 2007).

Variable Number of Tandem Repeats (VNTRs) is a useful method for typing genetically homogenous species (Van Belkum, 1999). This technique has been used to analyze the evolutionary history of plant and human pathogens (Dreo *et al.,* 2011; Bühlmann *et al.,* 2013; Hannou *et al.,* 2013). Likewise, other techniques such as MLST using housekeeping genes improve our understanding about bacterial phylogeny (Maiden *et al.,* 1998; Waleron *et al.,* 2008; McGhee *et al.,* 2011). In spite of genetic conservation of *rec*A, *rpo*S and *gro*EL genes, they are used for genetic relationships of enteric bacteria (McGhee *et al.*, 2002; Shrestha *et al.,* 2007).

Nonetheless, evaluation of genetic variation in *E. amylovora* strains has a significant role in epidemiological studies including pathogen spread, breeding programs, plant protection and quarantine measures (Puławska and Sobiczewski, 2012). Therefore, in order to find probable few differences between Iranian *E. amylovora* strains, the genetic analysis based on MLST using *rec*A, *rpo*S and *groEL* genes and also VNTR DNA marker was determined. Moreover, the combined data set was assessed for eventual microbial source tracking.

MATERIALS AND METHODS

Bacterial Strains

Forty *E. amylovora* strains were isolated from symptomatic plant materials in 2014 (Table 1). The remaining nine strains were related to our previous work (Taghdareh *et al.,* 2014). Isolation (Schaad *et al.,* 2001), phenotypic characterization (Geider *et al.,* 2009; Palacio-Bielsa *et al.,* 2012) and pathogenicity assay on immature pear slices (Mohammadi, 2010) were evaluated based on literature. Strain *E. amylovora* ATCC49946 (American Type Culture Collection) was used as positive control**.**

Molecular Confirmation at Species Level

Total genomic DNA was extracted based on Mohammadi (2010). For species identification, PCR amplification using A/ B and Ea71/ Ea71 primers was carried out twice for each set of primers as described in the literature (Bereswill *et al.,*1992; Taylor *et al.,* 2001) using an Applied Biosystems 2720 thermal cycler. The first primer pairs related to pEA29 plasmid of *Ea* and the second one corresponding to hypothetical protein in *Ea* genome. The PCR products were resolved on 1.2% (w⁄v) agarose gel, stained with green viewer (Genet Bio, Republic of Korea) and photographed under UV light.

Analysis of *E. amylovora* Strains Using MLST

Three housekeeping genes, *rec*A (Waleron *et al.,* 2002a), *rpo*S (Waleron *et al.,* 2002b) and *gro*EL (McGhee *et al.*, 2002, McGhee *et al*., 2011) were amplified for each strain. The PCR program included 32 amplification cycles of 94°C for 1 minute, 47°C for 1 minute (*rec*A), 55°C for 45 seconds (*rpo*S) or 60°C for 30 seconds (*gro*EL) and extension at 72°C for 2 minutes. PCR reactions were performed in a total volume of 25 µl containing 2.5 µl of PCR buffer, 200 µM of dNTPs, 2 mM $MgCl_2$, 10 pmole of each primer and 2.0 U of *Taq* polymerase (Cinnagen, Tehran). The PCR products were purified by QIAquick

Table 1. Name, host plant, sampling region and isolation year of studied *E. amylovora* strains.

Strain Name	Host	Geographical region	Year of isolation
SVA1-4	*Malus domestica*	Khorasan Razavi-Shandiz	2014
SVP1-4; SVPS1-3	*Pyrus communis*	Khorasan Razavi- zoshk	2014
KTQ1	*Cydonia oblonga*	Kalat	2014
KTPF1	*P. communis*	Kalat	2014
BPD	*P. communis*	Khorasan Razavi-Golbahar	2014
GKQ	*C. oblonga*	Khorasan Razavi-Golbahar	2014
GKPD	*P. communis*	Khorasan Razavi-Golbahar	2014
JPD	*P. communis*	Khorasan Razavi-Joghan	2014
JAL	*M. domestica*	Khorasan Razavi-Joghan	2014
JQ	*C. oblonga*	Khorasan Razavi-Joghan	2014
SAPA	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAPF	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAPD	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAPT	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAPS	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAP1	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAP2	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAP3	*P. communis*	Khorasan Razavi-SafiAbad	2014
SAA	*M. domestica*	Khorasan Razavi-SafiAbad	2014
AQ	*C. oblonga*	Khorasan Razavi-Akhlamad	2014
APF	*P. communis*	Khorasan Razavi-Akhlamad	2014
NKQ1-3; NKA	*C. oblonga*	Neyshabour-Kharv	2014
NBPR	*P. communis*	Neyshabour-Borj	2014
NBQ1	*C. oblonga*	Neyshabour-Borj	2014
NBAT	*M. domestica*	Neyshabour-Borj	2014
NBAL	*M. domestica*	Neyshabour-Borj	2014
MQ1	*C. oblonga*	Mashhad	2014
MPT	*P. communis*	Mashhad	2014
IrKh87	*P. communis*	Mashhad	2013
IrU23	*P. communis*	Uramia	2006
IrZa18	*M. domestica*	Zanjan	2005
IrS36	*Rosa* spp.	Semnan	2006
IrL53	*C. oblonga*	Lorestan	2007
IrGh59	*Crataegus* spp.	Ghazvin	2007
IrGh65	*P. communis*	Ghazvin	2007
IrIs11	*C. oblonga*	Esfehan	2012
IrIs12	*Crataegus* spp.	Esfehan	2012

PCR purification kit (Qiagen) and sequenced using an Automatic Sequencer 3730× (Macrogen, Republic of Korea). A set of comparable sequences for each gene from GenBank were aligned with MLST data, using ClustalX 1.81 (Thompson *et al.,* 1997). The phylogenetic analysis was performed using Maximum Likelihood method based on Tamura-Nei model in MEGA5 software (Tamura *et al.,* 2011). The related sequence of *Erwinia tasmaniensis*-ET1/99 was used as an out-group.

Genetic Analysis of *E. amylovora* Strains Using VNTR Sequences

The sequences located between the VNTR regions spreading in the *E. amylovora* genome were amplified with the primers VNTR4 (5'-CCCGGTGCTGC-3') and VNTR5 (5'- GCGGCGGCATG-3') reported by Hannou *et al.* (2013). For VNTR amplification, PCR program was done under the following conditions: 35 cycles of 94°C for 1 minute, 45°C for 1 minute, 65°C for 4

minutes; and a final extension step at 65°C for 15 minutes. The reproducibility of the fingerprinting patterns was checked three times. Two reference strains including ATCC49946 and CFBP1430 (The French Collection of Plant Pathogenic Bacteria) were used in this experiment.

RESULTS AND DISCUSSION

Characterization of *E. amylovora* Strains

All the *E. amylovora* strains isolated in 2014 were facultative anaerobic and formed mucoid yellow colonies on MM2Cu. The biochemical tests for hydrolysis of tween 20, nitrate reduction, production of fluorescent pigment on KB medium, levan production and acid production from D-xylose, odonitol, saccharose, D-mannose and D-arabitol agreed with those expected for *E. amylovora* species. In pathogenicity assay, symptoms including water-soaked lesions and tissue necrosis were observed on pear slices after artificial inoculation during one week. The results showed few differences among tested strains. Reference strain (ATCC49946) was applied as a positive control for comparison and negative control (sterile water) displayed no symptoms during the trial.

Identification Based on Specific Primers

Following amplification with primers A/B and Ea71/ Ea72, the 1,000 and 187 bp bands (Figure 1) expected for *E. amylovora,* were amplified in all strains. The same result was obtained for reference strains. No PCR product was amplified for negative control.

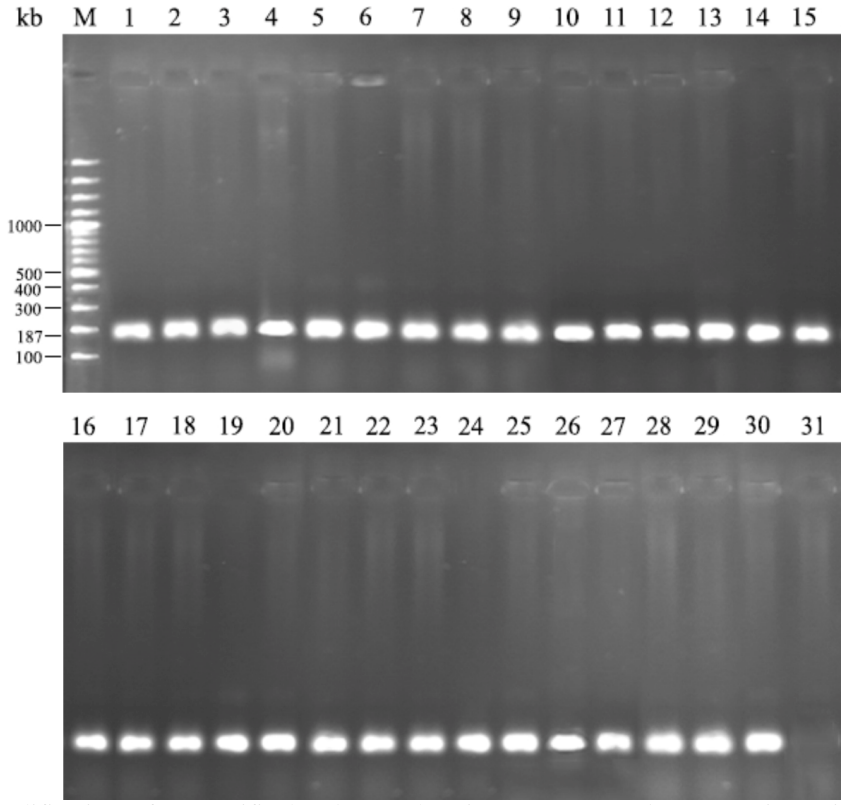

Figure 1. Amplification of a specific band (187 bp) in *Erwinia amylovora* strains using Ea71/Ea72 primers. M: 100 bp DNA ladder; 1: IrZ18; 2: IrS36; 3: IrL53; 4: IrGh59; 5: IrG65; 6: SVP1; 7: SVPS1; 8: KTQ1; 9: KTPF1; 10: JPD; 11: JQ; 12: SAPA; 13: SAPF; 14: SAPD; 15: SAPT; 16: SAP1; 17: SAA; 18: AQ; 19: NKQ2; 20: NKA; 21: NBPR; 22: NBAL; 23: NBAT; 24: GKQ; 25: JAL; 26: CFBP1430; 27: SVA1; 28: ATCC49946; 29: NBQ1; 30: MQ1, 31: Negative control.

Phylogenetic Analysis of MLST

Although some authors have concluded that the application of housekeeping genes in *E. amylovora* have no informative characters and may not reflect the genetic relatedness (McGhee *et al.,* 2002; Waleron *et al.,* 2008), a few nucleotide variations were found in *gro*EL sequences of *E. amylovora* strains in Michigan (McGhee *et al.,* 2011).

Eleven candidate strains (Figure 2) were chosen based on geographical regions and host plants. Sequence of each gene for Ea-ATCC49946, Ea-CFBP1430 and Ea-ATCC-BAA-2158 were retrieved from GenBank to construct an individual and combined phylogenetic tree. A BLAST analysis showed significant congruence (99–100% identity) among studied and reference strains and characterized our strains as *E. amylovora*. The Iranian strains were clustered together with North-American strain (ATCC49946) using the maximum likelihood phylogeny based on individual and concatenated sequence analysis. The obtained results of combined data sets (Figure 2) were supported by those published previously (Waleron *et al.,* 2008; McGhee *et al.,* 2011). The average mutation rate in Spiraeoideae-infecting strains is low. Alignment of partial *gro*EL sequences revealed one nucleotide difference in strain IrGh59 in comparison to other local and reference strains that is confirmed by further sequencing. This strain was isolated from *Crataegus* spp. in 2007 in Ghazvin Province, harboured pEA29 and carried a repeat sequence of 5 in *Pst*I fragment (Taghdareh *et al.,* 2014), but the reason of single nucleotide variation in IrGh59 is not clear. The determined sequences were deposited in the GenBank database under the accession numbers of KR270607 to KR270617 for *gro*EL, KR270618 to KR270628 for *rec*A and KR270629 to KR270639 for *rpo*S genes. Although the overall similarity in MLST data of *E. amylovora* strains is considerable, sequencing more housekeeping genes to search for eventual

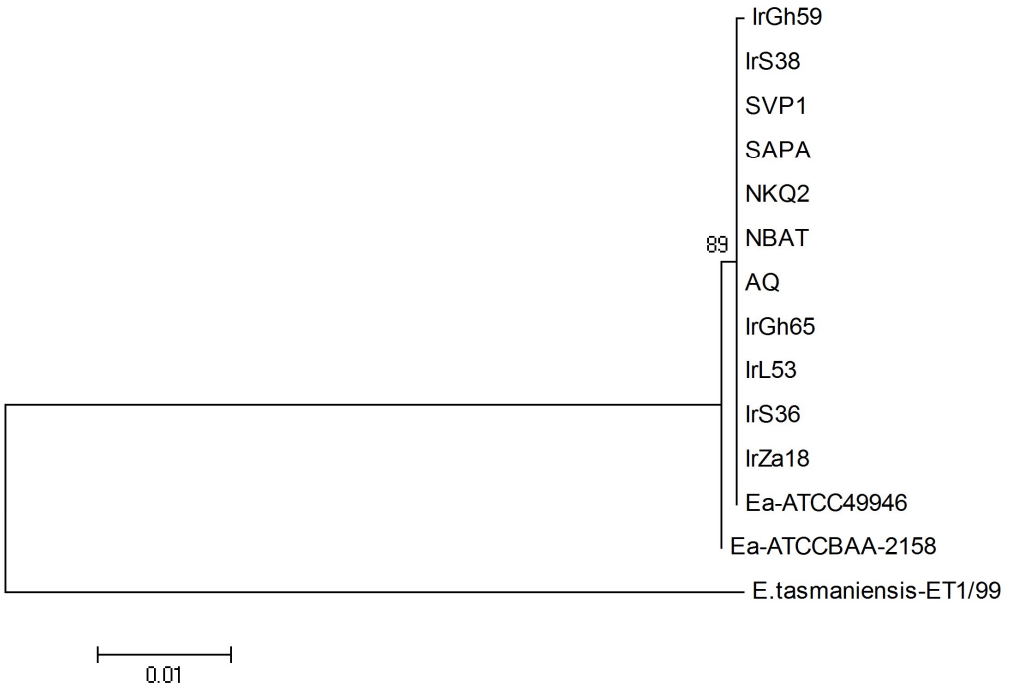

Figure 2. Phylogenetic tree of selective *Erwina amylovora* strains isolated from Rosaceae using concatenated gene portions of *rec*A, *rpo*S and *gro*EL based on maximum likelihood method. Bootstrap values after 1,000 replicates are expressed as percentages.

intraspecies variation is suggested.

Genetic Relatedness of Iranian *E. amylovora* Strains Based on VNTR Marker

Based on VNTR fingerprinting profiles, 50 strains of *E. amylovora* correlating to divers host plants and belonging to different parts of Iran especially eastern regions, showed homogeneity of their genomes. To achieve reliable data, all PCR reactions were repeated three times and specific patterns were achieved for tested strains. The overall results indicated that the fingerprinting profile of Iranian strains resembles to ATCC49946 American reference strain and is different from CFBP1430 European strain (Figure 3). This result was confirmed by the two VNTR primers used separately and simultaneously. There is no logical reason for this type of relatedness, because we have no official facts about source and spread of *E. amylovora* in neighboring countries and also disease entry to Iran.

Out of all molecular techniques used for *E. amylovora* separation, VNTR analysis along those techniques developed on the basis of *Ea* pan-genome, revealed high diversity between fire blight strains (Puławska and Sobiczewski, 2012). Among tested strains, two *E. amylovora* obtained from *Cydonia oblonga* out of 50 bacterial strains, generated different band patterns in comparison to basic profile. Identical ladder patterns were generated using VNTR4 for 49 isolates, whereas strain NBQ1 amplified some extra bands although very similar to the prevalent profile (Figure 3). The fingerprinting amplification obtained with VNTR5 primer, showed few variability in MQ1 genome (Figure 4), whereas NBQ1 strain was identical to the other strains' profile. Moreover, application of both VNTR 4 and 5 in the same PCR reaction revealed a specific profile and showed the genetic homogeneity of all strains except NBQ1(Figure5). These results are in agreement with Hannou *et al.* (2013) which reported few differences in Moroccan *E. amylovora* collection. No relation was seen

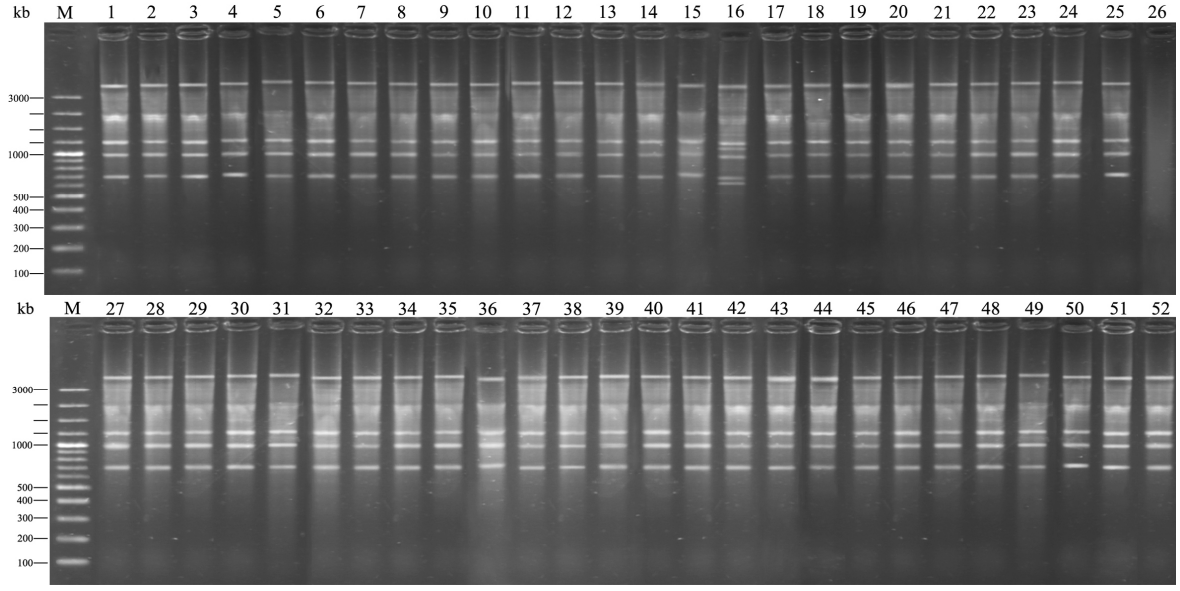

Figure 3. Fingerprinting patterns of Iranian *E. amylovora* strains using VNTR 4 primer. M: 100 bp DNA ladder; 1: IrZ18; 2: IrS36; 3: IrL53; 4: IrGh59; 5: IrG65; 6: SVP1; 7: SVPS1; 8: KTQ1; 9: KTPF1; 10: JPD; 11: JQ; 12: SAPA; 13: SAPF; 14: SAPD; 15: SAPT; 16: NBQ1; 17: SAA; 18: AQ; 19: NKQ2; 20: NKA; 21: NBPR; 22: NBAL; 23: NBAT; 24: APF; 25: JAL; 26: Negative control; 27: SVA1; 28: SVA2; 29: SVA3; 30: SVA4; 31: SVP2; 32: SVP3; 33: MPT; 34: SVP4; 35: SVPS2; 36: SVPS3; 37: BPD; 38: GKQ; 39: GKPD; 40: SAPS; 41: SAP2; 42: SAP3; 43: NKQ1; 44: NKQ3; 45: SAP1; 46: IrKh87; 47: IrS11; 48: IrS12; 49: IrU23; 50: ATCC49946; 51: MQ1, 52: CFBP1430.

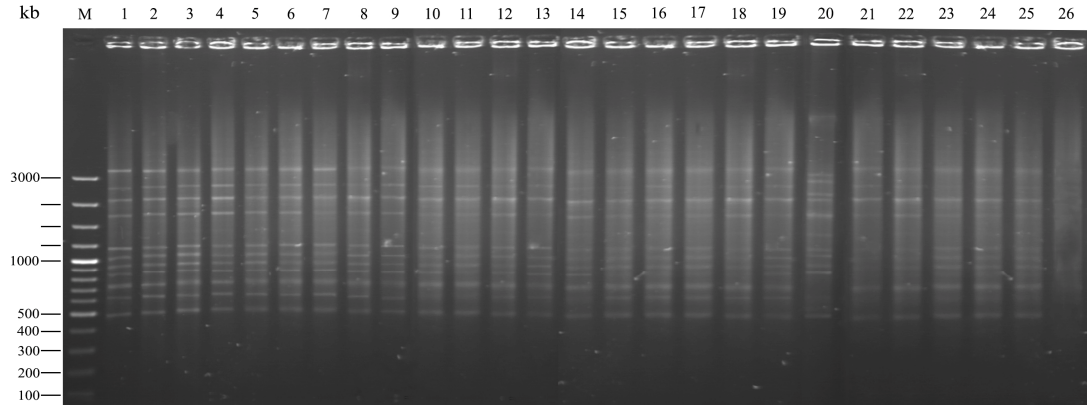

Figure 4. VNTR profiles analysis for selected *E. amylovora* strains by VNTR 5 primer. M: 100 bp DNA ladder; 1: IrZ18; 2: IrS36; 3: IrL53; 4: IrGh59; 5: IrG65; 6: SVP1; 7: SVPS1; 8: KTQ1; 9: KTPF1; 10; JPD; 11: JQ; 12: SAPA; 13: SAPF; 14: SAPD; 15: SAPT; 16: NBQ1; 17: SAA; 18: AQ; 19: NKQ2; 20: MQ1; 21: NBPR; 22: IrS12; 23: NBAT; 24: ATCC49946; 25: CFBP1430, 26: Negative control.

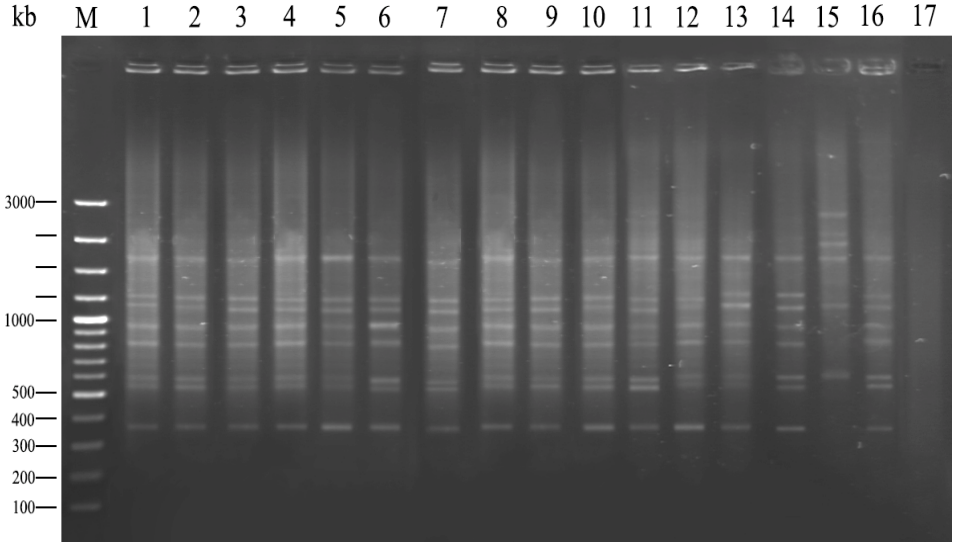

Figure 5. PCR fingerprinting patterns for representative *E. amylovora* strains using combination of VNTRs 4 and 5 primers. : 100 bp DNA ladder; 1: IrZ18; 2: IrS36; 3: IrL53; 4: IrGh59; 5: IrG65; 6: SVP1; 7: SVPS1; 8: KTQ1; 9: KTPF1; 10: JPD; 11: MQ1; 12: SAPA; 13: IrKh87; 14: ATCC49946; 15: NBQ1; 16: CFBP1430, 17: Negative control.

between the VNTR grouping and the host plant species or geographical regions.

Ten different regions have been sampled in Khorasan Razavi Province in 2014. The strain NBQ1 was isolated from 30 years old *Cydonia oblonga* plant cv. Neishabour collected from an ancient village named Borj in Neishabour, where it was allocated to cultivate quince species. Strain MQ1 was isolated from *Cydonia oblonga* cv. Esfahan from Mashhad region. Cultivar Esfahan is highly marketable compared to the first one and has been sent to different nurseries throughout the country. Apart from the foregoing, it seems that honey-bee movement with respect to blossom season may have been an important role in fire blight dispersal among different regions of Iran.

According to amplification profiles of VNTR in the current study, unique contamination source of fire blight disease in Iran is suggested. However, observed few genetic variations in Iranian *E. amylovora* strains may be related to other infection masses entry. Generally, it is concluded that

homogeneity is too high in Iranian *E. amylovora* strains, therefore application of more strains from different known sources can be assisted to gain more accurate results about genetic diversity of this pathogen and also disease distribution patterns.

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بررسی تنوع احتمالی بین جدایه های *Erwinia amylovora* ایران با استفاده از تایپینگ چند ژنی و آنالیز تکرارهای پیاپی

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چکیده

در مطالعه حاضر ارزیابی مولکولی ۵۰ جدایه *Erwinia amylovora* مربوط به مناطق مختلف ایران با استفاده از آنالیز توالی چند جایگاه ژنی (MLSA) و تعداد متنوع از تکرارهای پیاپی (VNTR) انجام گرفته است. درخت فیلوژنتیک ترکیبی ترسیم شده بر اساس توالی های نسبی ژن های ریکامبیناز A، سیگما فاکتور S و پروتئین شوک حرارتی GroEL، شباهت قابل توجه جدایه های ایرانی را نشان می دهد. فقط در جدایه IrGh59 بدست آمده از زالزالک مربوط به قزوین، تفاوت تک نوکلئوتیدی مشاهده گردید. در آنالیز VNTR، الگوی اثر انگشت یکسانی، مشابه جدایه مرجع ATCC49946 برای تمام جدایه های مورد مطالعه به استثنای NBQ1 و MQ1 بدست آمد، که گویای احتمال یکسانی منشاء آلودگی در ایران می باشد. افزون بر آن، جابجایی زنبور عسل با توجه به فصل گل می تواند نقش مهمی در پراکنش یکنواخت بیماری در کشور ما داشته باشد. جدایه های NBQ1 و MQ1 تولید کننده الگوهای VNTR متفاوت، به ترتیب از ارقام نیشابور و اصفهان گیاه به جدا شده اند. در این مورد خاص به طور قطع نمی توان اظهار نظر نمود. با این حال، ورود احتمالی منبع آلودگی دیگر، از کشورهای همسایه قابل بررسی است. در مجموع، الگوی انگشت نگاری VNTR به عنوان ابزار ارزیابی تفاوت ژنتیکی جمعیت های *E. amylovora* توصیه می گردد. همچنین بکارگیری تعداد جدایه بیشتر مربوط به منابع مختلف و مشخص، جهت دستیابی به نتایج دقیق تر در مورد تنوع ژنتیکی *E. amylovora* و الگوهای پراکنش بیماری سوختگی آتشی ضرورت دارد.