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Assessment of Genetic Diversity among *Xanthomonas arboricola* pv. *pruni* Strains Using *gyr*B Gene Sequencing and rep-PCR Genomic Fingerprinting in North Eastern Iran

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ABSTRACT

In the current study, the phenotypic and molecular properties of twenty-five strains obtained from cankerous tissues or leaf necrotic lesions of different stone fruits were evaluated in north-east of Iran . All strains studied were identified as *Xanthomonas arboricola* pv. *pruni* (Xap) based on phenotypic assays and confirmed by means of specific PCR at species and pathovar levels. All obtained strains were pathogenic under artificial inoculation and exhibited brittle necrotic spots on plum leaves of cultivar Santa Rosa under lab conditions. Then, the pathogenic *Xap* strains were subjected to molecular assays. In a phylogenetic tree constructed with *gyrB* sequences, no polymorphism was observed in this gene and Iranian *Xap* strains were clustered with the reference one in a separate group. The ERIC, BOX and REP primer sets generated reproducible genomic PCR profiles in tested strains and, based on combined data for all primers, a low genetic diversity among *Xap* strains was revealed. In order to achieve results that are more accurate, application of *Xap* strains from all geographical regions of Iran will be needed to prove little polymorphism observed in *Xap* population. The current contribution is the first report of molecular homogeneity of *Xap* strains that were collected from northeastern Iran.

Keywords: Bacterial leaf spot, Genetic similarity, Housekeeping gene, Repetitive genomic elements.

INTRODUCTION

Xanthomonas arboricola has the extended host range from a set of various hosts (Fischer-Le Saux *et al.*, 2015). Among its pathovars, *X. a.* pv. *pruni* (Xap) is a severe pathogen in stone-fruit-growing regions of the world (Bergsma-Vlami *et al.*, 2012). Although the natural hosts of *Xap* are *Prunus* species, almond and cherry are also reported (Jami *et al.*, 2005; Palacio-Bielsa *et al.*, 2010).

Bacterial spot caused by *Xap* created fruit and leaf lesions and induced necrotic areas on tree branches as canker (Jacques *et al.*, 2016). These lesions sometimes can be confused with injuries caused by other microorganism or abiotic factors such as wounds caused by cultural practices (Lamichhane, 2014; Rosello *et al.*, 2012). Therefore, the diagnosis approaches are necessary to confirm the precise detection of *Xap* and proper management solutions. Considerable economic losses occurred when the disease reduced orchard yields and fruit marketability (Stefani, 2010; Lamichhane, 2014).

The disease has currently been reported from all continents including America, Africa, Asia, Europe and Oceania (EFSA, 2014). Further disease distribution has only been controlled by strict phytosanitary

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legislation of the European Union (EPPO, 2006), while other regional plant protection organizations have considered no quarantine concern (Lamichhane, 2014). The latent contaminated propagative plant materials, rain, and infected pruning tools probably represent the highest risk of introduction and spread of the disease (Loreti *et al.*, 2015).

Although the presence of *Xap* causes severe losses and limits the production of susceptible cultivars of stone fruits in several countries (Stefani, 2010, Palacio-Bielsa *et al.*, 2012), bacterial spot caused by this pathogen is not an economic disease in Iran, yet. As the spread and loss of *Pseudomonas syringae* pv. *syringae* on stone fruits in Iran has priority compared to *Xap*, little has been done on *Xap* survey. Bacterial canker and leaf spot have been previously reported with a restricted distribution on *Prunus* crops in northern parts of Iran (Jami *et al.*, 2005; Mahmoudi *et al.*, 2011).

An integrated approach that includes necessary measures to avoid *Xap* entrance in healthy areas by application of resistant host plants, as well as the copper compounds usage and establishment of efficient practical cultivation methods has been suggested in order to manage bacterial spot (Stefani, 2010).

Investigations of genomic variability could provide useful information in breeding programs for resistance and plant protection. The intra-pathovar diversity of *Xap* is estimated low on a wide range of *Prunus* species (Hajri *et al.*, 2012). Boudon and co-workers (2005) analyzed a collection of *Xap* strains belonging to three different countries using the intergenic transcribed spacer region and housekeeping genes and observed low level of variation. Barionovi and Scortichini (2008) applied Box-PCR assay on *Xap* strains collected from Australia, Italy, and Spain, and no polymorphism was detected based on their results. The *Xap* strains isolated from peach orchards in Japan are genetically nearly homogeneous by means of rep and ISSR primers (Kawaguchi, 2014). In another study, strains of *Xap* obtained from peach and plum orchards in northeastern Italy exhibited slight genetic variation based on rep-PCR analyses (Giovanardi *et al.*, 2017).

Little is known about the possible heterogeneity and genetic relationship among strains of *Xap* infecting *Prunus* species in Iran. During late spring of 2015, a survey was conducted in stone fruits orchards in northeastern Iran for the presence of *Xap* strains. Detection was performed by phenotypic assays and confirmed by specific PCR in species and pathovar levels. The principal objective of this study was to characterize the genetic diversity within Iranian *Xap* strains using *gyrB* sequencing and Repetitive-sequence typing for the first time.

MATERIALS AND METHODS

Bacterial Strains

During late spring and early summer of 2015, symptomatic samples were collected from plum, peach, apricot and sweet cherry cultivars in Khorasan Razavi Province located in northeastern Iran. Bacterial isolation was performed on yeast extract dextrose calcium carbonate agar medium (Stolp and Starr, 1964). Yellow, mucoid colonies with entire margin were selected.

Phenotypic Assays

List of *Xap* strains studied is summarized in Table 1. Phenotypic tests including fermentative metabolism, oxidase and catalase activity, H_2S production from cysteine, growth on 1% TetraZolium Chloride (TZC) and 5% NaCl, hydrolysis of starch, gelatin, casein, and esculin, and levan production were performed according to literatures (Palacio-Bielsa *et al.*, 2012; Schaad *et al.*, 2001).The assimilation of carbon sources such as sucrose, raffinose, and lactose were tested on the basal medium of Ayers *et al.* (1919). The quinate

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Table 1. Name, host plant, and sampling regions of studied *Xanthomonas arboricola* pv. *pruni* strains obtained from different *Prunus* spp. in north eastern Iran.

Strain Name	Host	Geographical region
ML37	Apricot	Mashhad
MB29	Apricot	Mashhad
MB33	Plum	Mashhad
MB34	Plum	Mashhad
MB35	Plum	Mashhad
Ml41	Peach	Mashhad
TB31	Plum	Khorasan Razavi-Torghabeh
TB47	Plum	Khorasan Razavi-Torghabeh
TL34	Sweet cherry	Khorasan Razavi-Torghabeh
TL49	Apricot	Khorasan Razavi-Torghabeh
TB50	Apricot	Khorasan Razavi-Torghabeh
ShL45	*Plum*	Khorasan Razavi-Shandiz
ShB51	Plum	Khorasan Razavi-Shandiz
ShB32	Sweet cherry	Khorasan Razavi-Shandiz
CB19	Apricot	Chenaran
NB28k	Plum	Neyshabour
NB28	Plum	Neyshabour
NB26	Plum	Neyshabour
NB27	Plum	Neyshabour
NB14	Sweet cherry	Neyshabour
NB16	Sweet cherry	Neyshabour
NB21	Sweet cherry	Neyshabour
QL36	Apricot	Ghochan
QB24	Apricot	Ghochan
QB24k	Apricot	Ghochan
QB22	Apricot	Ghochan
QB15	Sweet cherry	Ghochan
QB18	Peach	Ghochan

metabolism was evaluated using succinate quinate medium (Lee *et al.,* 1992). In addition, xanthomonadin pigment was purified and analyzed by spectrophotometry (Chen, 2002). Reference strain of ICMP7485 and distilled water were used as positive and negative controls, respectively, in all assays.

Molecular Characterization

The accuracy of phenotypic assays was confirmed by specific PCR. Total genomic DNA from pure bacterial cultures was extracted by the method of Scortichini *et al*. (2002). The identity of *Xap* strains was performed by XarbQ F/R (Pothier *et al.*, 2011) and XapY17F/ XapY17R primers (Pagani, 2004) at species and pathovar levels, respectively, using an Applied Biosystems 2720 thermal cycler. 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.

Pathogenicity Test

Pathogenicity of studied strains was determined using a detached leaf assay (Randhawa and Civerolo, 1985). Briefly, young leaves of plum seedlings cv. Santa Rosa were surface sterilization with 70% ethanol for 40 s and rinsed three times in sterile water. These leaves were then infiltrated by bacterial suspension of 10^7

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CFU mL^{-1} and incubated on 5% water agar at 25ºC for 2 weeks under a 16 hour photoperiod. Experiments were repeated two times with five repetitions. Negative controls consisted of leaves infiltrated with sterile water, whereas a *Xap* strain (ICMP7485) was used as a positive control.

Sequence Analysis of *gyrB* Gene

Amplification of *gyrB* gene was applied with the primers reported by Essakhi *et al.* (2015). Fifteen strains were selected based on host plant and region (one from each *Prunus* sp. in each region). The amplicons were purified with the QIA quick PCR purification kit (Qiagen, USA) and sequenced in both directions by commercial service (Macrogen Inc., Korea).

Nucleotide sequences were subjected to alignment using Clustal X 1.83 (Thompson *et al*., 1997). A phylogenetic tree was generated based on neighbor-joining algorithm and presented using software MEGA 5.0 (Tamura et *al*., 2011) with 1000 replicates for bootstrap analysis.

The rep-PCR Genomic Fingerprinting

In order to study genetic relationships among *Xap* strains, polymerase chain reactions were carried out using ERIC, Rep (Versalovic *et al*., 1991) and Box (Louws *et al.*, 1994) primers following procedures described in the literatures for candidate strains. After electrophoresis, the size of the PCR products was estimated using a 3,000 bp DNA ladder (Small Bio, Small tool). A digital image of each gel was subjected to analysis using the Photo-Capt software (Vilber Lourmat Co. Deutschland , Eberhardzell, Germany). The scores obtained from each PCR method were combined for analysis. Final dendrogram was constructed with the UPGMA algorithm using MVSP (Multivariate System Package, version 3.131) software.

RESULTS

Characterization of *X. arboricola* Strains

Twenty-five mucoid yellow-pigmented colonies obtained from cankerous tissues or leaf necrotic lesions of stone fruits in northeastern Iran were obligate aerobe, oxidase negative, and catalase positive. All of them produced levan and H_2S from cysteine and showed no growth on TZC 0.1%. Moreover, absorption spectra of Xanthomonadin pigment extracted from strains were similar to that of reference strain of ICMP7485 and were determined in the range of 441-447 nm. All mentioned phenotypic tests showed that the isolated strains from stone fruit trees belonged to *Xanthomonas* genus.

Production of deep green color around bacterial colonies in succinate quinate medium indicated quinate metabolism of the strains, which is the specified character of *X. arboricola* species. Other tests including growth at 35ºC, esculin hydrolysis, and acid production from sucrose were positive, while metabolism of raffinose, lactose, and hydrolysis of starch recorded negative. Some tests including hydrolysis of gelatin and casein and tolerance to 5% sodium chloride were variable among the strains tested.

Molecular Detection of the *Xap* Strains and Pathogenicity Test

Amplification of specific 402 bp bands from all bacterial strains was generated using *qum*A specific primers that confirmed the positive reaction of tested strains in succinate quinate medium and determined all strains as *X. arboricola* at species level. Moreover, the expected 943bp PCR-amplified fragment obtained with the XapY17 primers confirmed belonging of the strains tested to pruni pathovar.

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In pathogenicity test, symptoms appeared as confluent water soaking lesions that became brown and exhibited brittle necrotic spots in plum leaves, similar to positive control, ICMP7485. None of the control plants inoculated with sterile water showed signs of the disease.

Phylogenetic Analysis of *gyrB*

Fifteen representative strains belonging to different *Prunus* spp. were subjected to *gyrB* sequencing. A comparative BLAST analysis of the *gyrB* sequences with those retrieved from GenBank (Fischer-Le Saux *et al.*, 2015; Essakhi *et al.*, 2015) revealed that the studied strains belong to *Xap*. All native strains clustered tightly together with known different *Xap* strains (Figure 1). Strains belonging to close pathovars including *juglandis* and *corylina* were placed in a separate clade. The determined sequences were deposited in the GenBank database under the accession numbers of MK355464 to MK355478.

Repetitive Element Palindromic PCR

The rep-PCR assay, which targets the highly conserved DNA sequences present in

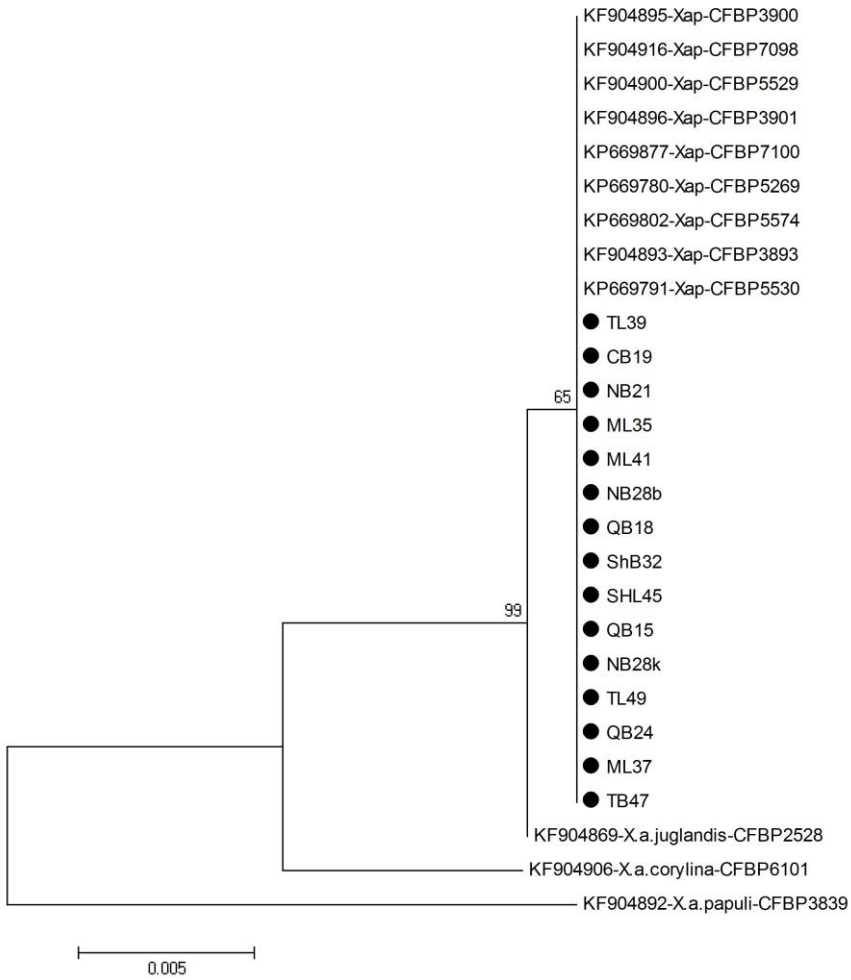

Figure 1. Phylogenetic tree constructed by neighbor-joining analysis of partial *gyr*B gene sequences of *Xanthomonas arboricola* pv. *pruni* strains obtained from stone fruit trees of north eastern, Iran (shown by ●), other sequences retrieved from GenBank (shown by accession number). Bootstrap values are observed on the main branches.

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bacterial species, is considered as a discriminatory technique to determine taxonomic diversity (Rademaker *et al.*, 2000). The ERIC, BOX and REP primer sets generated reproducible genomic PCR profiles consisting of bands of approximately100 to 3,000 bp for fifteen representative *Xap* strains (Figure 2). The number of fragments varied from 7 to 10 in rep-PCR assays. The UPGMA analysis was carried out with the strains tested and, based on combined data for all primers, the constructed dendrogram highlighted two clustering groups, which is shown in Figure 3. The rep-PCR outcomes placed the majority of the *Xap* strains with ICMP7485 inside clade one, which consisted of two subgroups. The subgroup A included all strains obtained from Neyshabour Region. The other subgroup consisted of strains isolated from different plant tissues including leaves and branches, from various *Prunus* spp. and sampling regions. The eight *Xap* strains with the extra bands in BOX and REP reactions were placed in clade two. All fragments generated by ERIC primers were monomorphic (Figure 2).

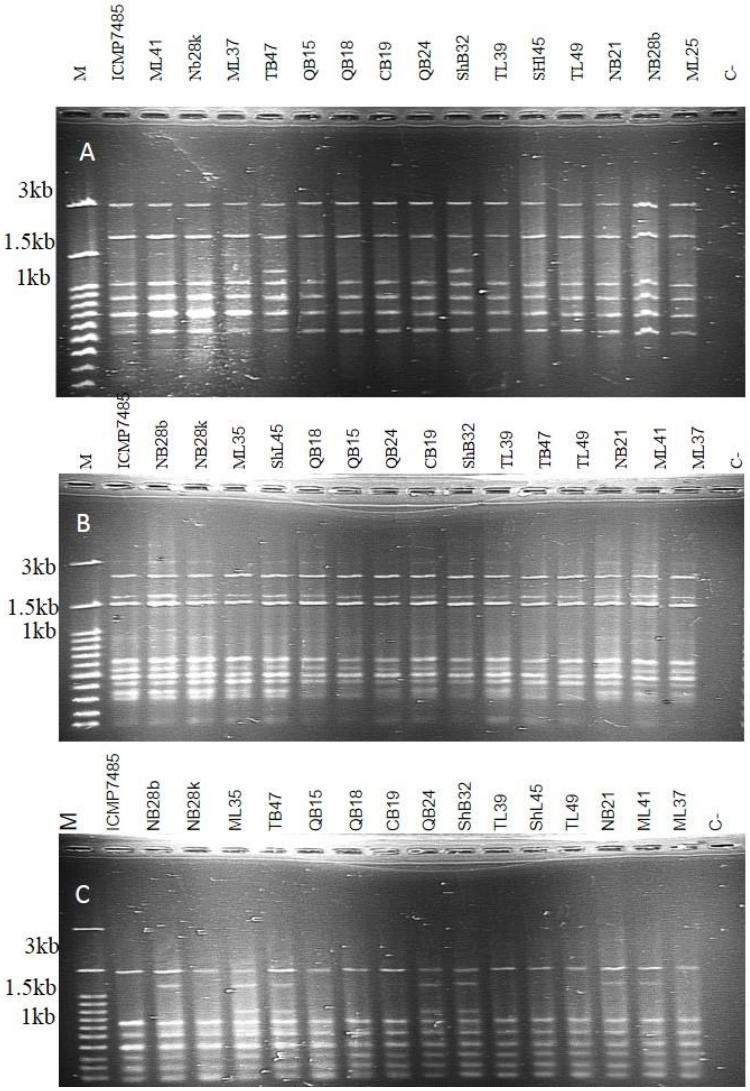

Figure 2. Fingerprinting patterns of *Xanthomonas arboricola* pv. *pruni* strains obtained from stone fruit trees of north eastern Iran using BOX (A), ERIC (B) and REP (C) primers. (M): 3kb DNA ladder, (C-): Negative control.

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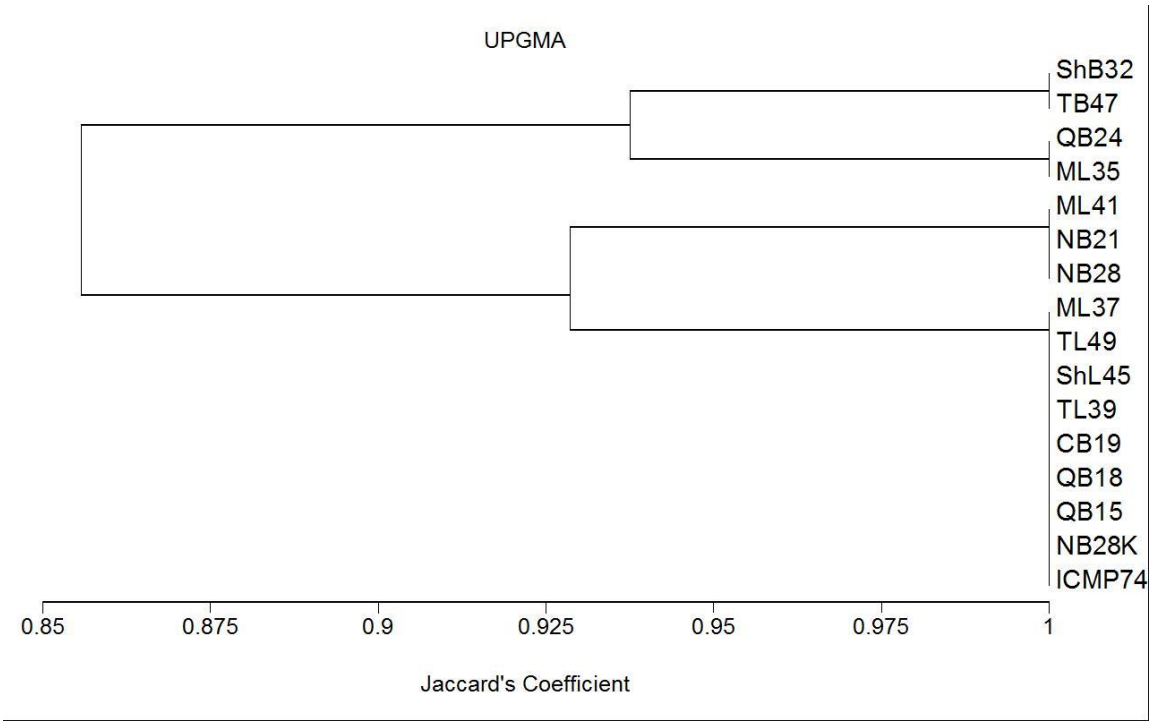

Figure 3. Dendrogram of *Xanthomonas arboricola* pv. *pruni* strains based on combined ERIC, BOX, and, REP-PCR fingerprinting constructed by UPGMA algorithm. *X. a.* pv. *pruni* ICMP7485 was used as a reference strain.

DISCUSSION

Xanthomonads, as plant pathogens, can infect several monocot and dicot plant species in different families (Vauterin *et al.*, 2000). The species belonging to this genus comprise pathogenic strains distributed in different pathovars (Rodriguez-R *et al.*, 2012) that interact with intraspecies variants of hosts.

Disparate populations of nonpathogenic Xanthomonas strains from different plants are also reported that do not group with the pathogenic strains of the plant host from which they were obtained and most of them are categorized as *X. arboricola* using phylogenetic analyses (Vauterin *et al.*, 1996; Essakhi *et al.*, 2015). Therefore, precise diagnostic tools are necessary to confirm the bacterium presence in infected tissues and to differentiate pathogenic *X. arboricola* strains from nonpathogenic ones (Garita-Combronero *et al.*, 2017).

The stone fruit quarantine pathogen, *Xap*, is one of the most economically important pathovars in *X. arboricola*, which can significantly decrease crop yield, and result in orchard loss (Pothier *et al.*, 2011). The bacterial spot caused by this pathogen is more severe in humid areas with a warm temperature in the range of 19 to 28ºC (Morales *et al.*, 2017). Although northeastern Iran is not wet enough to allow *Xap* for extended infection on stone fruit species, frequent rainfall occurred during spring and summer of 2015 and facilitated the pathogen spread in our zone in that time.

In the present study, the obtained colonies were yellow, mucous, and round with entire margin on YDC medium following isolation from symptomatic tissues of stone fruit samples. Identification of strains was performed using preliminary assays based on phenotypic observations, which matched well with previous studies (Palacio-Bielsa *et*

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al., 2012; Schaad *et al.*, 2001). Observed variation in some phenotypic test consisting of tolerance to 5% sodium chloride and hydrolysis of gelatin and casein were in line with prior report from Iran (Jami *et al.*, 2005). Quinate metabolism of the strains differentiated *X. arboricola* from other Xanthomonas spp. (Lee *et al.*, 1992).

Phenotypic assays are not discriminative enough to adequately identify *Xap* strains. Therefore, bacterial colonies with typical morphology of *Xap* were subjected to specific PCRs. The accurate identification of *X. arboricola* strains was confirmed using species- specific primers corresponding to *qumA* gene sequence encoding for quinate metabolism (Pothier *et al.*, 2011). Based on pathovar specific primers, targeting a specific 943-bp fragment of a putative ABC transporter ATP-binding protein, described in Pagani (2004), the current research strains were characterized as pathovar pruni. Although cross-reaction of second primers with other *X. arboricola* pathovars encompassing *X. arboricola* pv. *corylina*, *celebensis* and *poinsettiicola* were shown before (Pothier *et al.*, 2011), none of these pathovars were reported from *Prunus* spp. However, the isolation produced typical *Xap* colonies that Y17-PCR and pathogenicity assays confirmed that processes and this is in line with the detection and identification diagram of *Xap* that was presented by Loreti *et al.*, (2015).

Pathogenicity of *Xap* strains was recorded as necrotic spot on infiltrated test leaves. Infiltration in the leaves using a needleless syringe proved as an efficient technique according to Socquet-Juglard *et al*. (2012). According to Garita-Combronero *et al*. (2016), nonpathogenic strains of *Xap* were starch positive, while our strains showed negative reaction in starch medium and caused expected symptoms on plum leaves in the lab assy.

As described in literature, *X. arboricola* encompasses nine pathovars with a diverse range of biotic relationships (Fischer-Le Saux *et al.*, 2015). The main pathovars of this species including *pruni*, *corylina*, and *juglandis*, which cause disease in nut, stone fruit trees, and almond (Palacio-Bielsa *et al.*, 2010), are considered as highly phylogenetically relevant groups based on multilocus sequence typing (Fischer-Le Saux *et al.*, 2015). The other strains of the species, either weakly or not pathogenic, are phylogenetically heterogeneous (Garita-Cambronero *et al.*, 2018). The specified difference among these three most virulent pathovars of *X. arboricoa* is related to their proprietary interaction of the bacteria with the host plant, which creates a suitable condition for their survival and duplication (Jacques e*t al.*, 2016).

Several phylogenetic and molecular methods have been used to survey *Xap* population biology worldwide. Analysis of the partial sequences of housekeeping genes such as *atpD*, *efp* and *glnA* had the ability to separate *Xap* strains from close relative pathovars (Boudon *et al.*, 2005; Fischer-Le Saux *et al.*, 2015). Based on literature, the *gyrB* phylogeny clearly provided the distinctiveness of *X. arboricola* at pathovar levels (Parkinson *et al.*, 2009). No genetic variation was characterized among the *Xap* strains tested within the housekeeping gene sequence, in agreement with other studies (Boudon *et al.*, 2005; Giovanardi *et al.*, 2017). Subsequent studies showed minor polymorphism in *Xap* population, but further indicated its monomorphic nature (Fischer-Le Saux *et al.*, 2015).

In rep-PCR assay, a high genetic similarity (92.5%) was observed for the studied strains, which revealed a low diversity among *Xap* strains of north-eastern Iran and is supported by previous researches (Boudon *et al.*, 2005; Giovanardi *et al.*, 2017). Application of integron gene cassette array and ISSR-PCR also showed low polymorphism amongst *Xap* strains (Barionovi and Scortichini 2008; Kawaguchi, 2014). The molecular patterns of rep-PCR observed in *Xap* strains presented no relationship with host and the geographical region from which the strains were isolated. The low genetic variability might be due to the limited genetic diversity

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of the host species (Hajri *et al.*, 2012) and high nucleotide identity amongst all members of this pathovar that is proved by whole-genome analyses (Garita-Cambronero *et al.*, 2017). However, other techniques such as Multilocus Variable number of tandem repeats (MLVA) scheme clearly classified 25 *Xap* strains from a world collection into 23 haplotypes (Lopez-Soriano *et al.*, 2016).

CONCLUSIONS

Summarizing the results, although the studied housekeeping gene sequence was identical in *Xap* strains obtained from stone fruit trees in 2015, repetitive-sequence typing that can encompass the whole bacterial genome determined low diversity amongst the strains tested and may be considered as an effective fingerprinting method. Nevertheless, there is a need to explore further *Xap* strains from all cultivating areas in Iran in order to achieve reliable results. Moreover, usage of MLVA that show high discriminatory capability in *Xap* differentiation is recommended in future studies. This is the first report that evaluates Iranian *Xap* strains infecting *Prunus* species by molecular assays and reports their genetic homogeneity.

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ارزیابی تنوع ژنتیکی جدایه های *Xanthmonas arboricola pv. pruni* با استفاده از توالی یابی ژن *gyrB* و انگشت نگاری ژنومی rep-PCR در شمال شرق ایران

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چکیده

در مطالعه حاضر، ویژگی های فنوتیپی و مولکولی ۲۵ جدایه بدست آمده از شانکر و لکه نکروتیک برگی هسته داران مختلف در شمال شرق ایران ارزیابی گردید. تمامی جدایه های مورد مطالعه بر پایه سنجش های فنوتیپی و واکنش زنجیره ای پلیمراز اختصاصی گونه و پاتووار به عنوان Xanthomonas arboricola pv. pruni (Xap) شناخته شدند. تمامی جدایه های بدست آمده تحت مایه زنی مصنوعی در آزمایشگاه بیماریزا بوده و لکه های شکننده نکروتیک را بر روی برگهای آلو زقم سانتاروزا نشان دادند. سپس جدایه های Xap بیماریزا تحت آزمایشات مولکولی قرار گرفتند. در درخت فیلوژنتیک ترسیم شده توسط ژن gyrB ، چندشکلی در این ژن در جدایه های ایرانی Xap مشاهده نگردید و جدایه ها به همراه جدایه استاندارد در یک گروه قرار گرفتند. جفت آغازگرهای REP,REIC و BOX الگوهای PCR تکرارپذیری را در جدایه های مورد بررسی ایجاد نموده و بر اساس داده های ترکیبی مربوط به هر سه آغازگر، تنوع ژنتیکی اندکی بین جدایه های Xap مشخص گردید. به منظور دستیابی به نتایج دقیق تر، بکارگیری جدایه های Xap بیشتر از تمام مناطق جغرافیایی ایران جهت اثبات چند شکلی اندک مشاهده شده در جمعیت Xap ضروری خواهد بود. مطالعه حاضر اولین گزارش از همگنی مولکولی جدایه ها ی Xap جمع آوری شده از شمال شرق ایران می باشد.

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