Assessment of Genetic Diversity among *Xanthomonas arboricola* pv. *pruni* Strains Using *gyrB* 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...
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, H₂S 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
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.
CFU mL\(^{-1}\) and incubated on 5% water agar at 25\(^{\circ}\)C 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\(_2\)S 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 35\(^{\circ}\)C, 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 qumA 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.
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

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**Figure 1.** Phylogenetic tree constructed by neighbor-joining analysis of partial gyrB 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.
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 approximately 100 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.
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
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, celtensis and poinsettii are 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-Combroner 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 assay.

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. arboricola is related to their proprietary interaction of the bacteria with the host plant, which creates a suitable condition for their survival and duplication (Jacques et 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 جدایی های Xanthomonas arboricola pv. pruni با استفاده از توآلی باید و انکشت تکاری زنومی gyrB و rep-PCR در شمال شرق ایران

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چکیده

در مطالعه حاضر، برگی هسته داران مختلف در شمال شرق ایران ارژیابی گردید. تمامی جدایی های مورد مطالعه بر پایه سنجش های فوتونی و واکنش زنجبیر یک بیماری اختصاصی گونه و پاتوپور به عنوان مشابهت هسته ای. تمامی جدایی های گردید آمده Xanthomonas arboricola pv. pruni (Xap) تحت مایه زنی مصنوعی در آزمایشگاه بیماری‌زا یافته و لکه‌های شکننده نکروتیک را بر روی برگ‌های آلم زمین مصنوعی در آزمایشگاه بیماری‌زا نشان دادند. میزان تعداد Xap و Xap تحت آزمایشات مولکولی قرار گرفتند. درخت فیلوژنیک ترسیم شده توسط ZnB، جنرال گروه در این Zn در جدایی های ایرانی مشاهده گردید. و جدایی ها به همراه جدایی استاندارد در یک گروه قرار گرفتند. نتایج آغازگردهای PCR و BOX و و REP، REIC تکرارپذیری و در جدایی های مورد بررسی ایجاد نموده و بر اساس داده های تکریپ مربوط به هر یک آغازگر، نوع زنینی اندکی بین جدایی های گردید. به منظور دستیابی به نتایج دقیق تر، با کارگری جدایی های Xap به‌-minus از تمام مناطق جغرافیایی ایران جهت انتخاب یک شکل اندک مشاهده شده در جمعیت Xap در صورتی خواهد بود. مطالعه حاضر اولین گزارش از همگی مولکولی جدایی های Xap باشد. جمع آوری شده از شمال شرق ایران می‌باشد.