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Diversity of Iranian isolates of *Ralstonia solanacearum*

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Summary. During 2007–2009, 170 bacterial isolates were obtained from wilting potato and tomato plants from Iran. Specific polymerase chain reaction (PCR) analyses identified all the isolates as *Ralstonia solanacearum*. Diversity among the Iranian isolates was determined by a study based on biovar determination, pathogenicity, repetitive sequence PCR and phylotype classification. On the basis of genotypic, phenotypic and pathogenic characteristics two distinct clusters obtained. Cluster I comprised all biovar 2 isolates and cluster II comprised all biovar N2 (2T) isolates. Phylotype-specific multiplex PCR (pmx-PCR) indicated that all biovar 2 and N2 belong to phylotype II. This is the first report of phylotype determination of biovars of *R. solanacearum* with pmx-PCR in Iran.

Key words: bacterial wilt, Iranian biovars, genetic diversity.

Introduction

Bacterial wilt of potato is caused by *Ralstonia solanacearum* (E.F Smith) (Yabuuchi *et al,*. 1995) and is one of the most destructive and widespread bacterial diseases of potato in the world (Hayward, 1991). *Ralstonia solanacearum* is a heterogeneous species (Hayward, 1994) and historically, it has been identified by its host range (defining races) (Buddenhagen *et al*., 1962; He *et al*., 1983) and catabolic properties (defining biovars) (He *et al*., 1983; Hayward, 1994). Potato isolates of *R. solanacearum* usually belong to biovar 1/ race 1, biovar 2/ race 3 and biovar N2 (2T). Based on its host range, biovar N2 is equivalent to race 3 or race 1 (Hayward 1994; Horita and Tsuchiya, 2001). Biovar N2 is a new variant (a tropical variant, 2T) that was discriminated from biovar 2 in some phenotypic traits (French *et al*., 1993; Gillings and Fahy, 1993; Marin and El-Nashaar, 1993). Although the identification based on biochemical tests proved to be useful in the past and is currently accepted, it is very time-consuming (Gillings and Fahy, 1993; Villa *et al*., 2005; Cruz *et al*., 2008). Therefore, DNA-based analyses have been used to increase our understanding of the genetic relatedness among *R. solanacearum* isolates, on the basis of restriction fragment length polymorphisms (RFLP) (Cook *et al.,* 1989) and 16S rRNA gene sequences (Li *et al*., 1993; Taghavi *et al.,* 1996). *Ralstonia solanacearum* can be subdivided into two major groups, designated division I and II. Division I comprises biovars 3, 4 and 5 and division II comprises biovars 1, 2 and N2. Fegan and Prior (2005) analyzed sequence data of the internal transcribed spacer (ITS) region to develop a phylogeny-based scheme. This classification yielded four genetic or phylotype groups of the *R. solanacearum* species complex. Each phylotype correlated with the geographic origin of isolates. Phylotype I includes Asian isolates; phylotype II isolates from America, phylotype III from Africa

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and phylotype IV isolates from some Asian countries (Indonesia, Philippines, Japan, Korea) and Australia (Fegan and Prior, 2005; Jeong *et al*., 2007). This classification scheme was recently confirmed by using a whole-genome microarray approach (Guidot *et al*., 2007). *Ralstonia solanacearum* biovar N2 isolates belong to phylotype II, III and IV (Fegan and Prior, 2005; Villa *et al*., 2005; Xu *et al*., 2009; Horita *et al*., 2010).

Bacterial wilt has been one of the most important diseases of potato and tomato in Iran for a long time. Outbreak zones of this pathogen are still expanding, threatening the production of potato and tomato in different parts of the country. Biovar 2 isolates of *R. solanacearum* were identified as the causal agent of bacterial wilt of potato in Iran. Recently, three *R. solanacearum* isolates from potato have been reported as biovar N2 (Bahar and Danesh, 1988; Bagheri and Taghavi, 2001; Maghooli *et al*., 2004; Taghavi *et al*., 2004; Irandoost *et al*., 2007; Nouri *et al*., 2008).

The present study was conducted to investigate the relationships among Iranian isolates of *R. solanacearum* in the main potato and tomato growing areas of this country, to evaluate the phenotypic and genotypic characteristics, to characterize the phylotypes of isolates and to determine their geographic distribution.

Materials and methods

Bacterial isolates, isolation and conditions

From 2007 to 2009, 162 bacterial isolates from wilted potato plants were obtained from some provinces of Iran (Figure 1). Six isolates previously collected (2004) from wilted potato and tomato plants (isolates 145–150) and two reference isolates of biovar N2 (isolate 153, 154) and 2 (isolate G1, G2) were also used in this study. The main features of the bacterial isolates used in this study, are listed in Table 1.

Physiological and biochemical tests

Physiological and biochemical tests included gram reaction, Kovac's oxidase, catalase, Tween 80 and starch hydrolysis, catalase and oxidase production, nitrate reduction, gelatin liquefaction, melanin formation (tyrosinase) and pectolytic activity tests, and were carried out according to

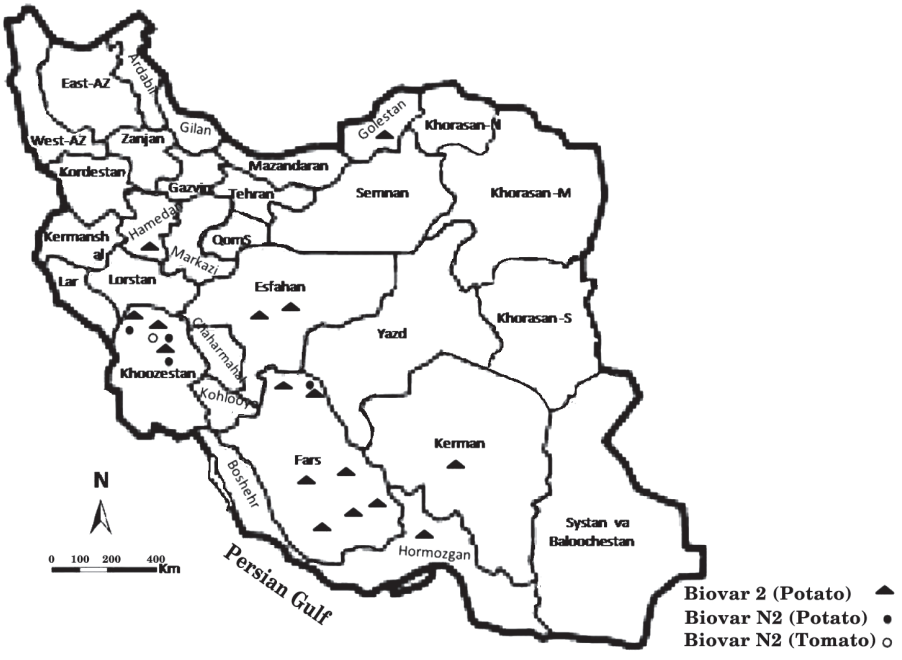

Figure 1. Locations in Iran where *Ralstonia solanacearum* isolates were collected.

Table 1. Characteristics of *Ralstonia solanacearum* isolates used in this study.

Isolate	Host	Location	Year	Season	Biovar
1–42	Potato	Fars/Eghlid	2007	Autumn	2
43–50	Potato	Fars/Norabad	2008	Summer	2
51–54	Potato	Fars/Darab	2008	Summer	2
55–62	Potato	Fars/Dehbid	2007	Autumn	2
63–70	Potato	Fars/Maharlo	2008	Spring	2
71–86	Potato	Hamedan/Bahar	2008	Summer	2
87–103	Potato	Kerman/Jiroft	2009	Spring	2
104–105	Potato	Khoozestan/Dezful	2009	Summer	2
106	Potato	Khoozestan/Andimeshk	2009	Summer	2
107–110	Potato	Fars/Fasarod	2008	Autumn	2
111–112	Potato	Esfahan/Dorche	2007	Autumn	2
113–115	Potato	Esfahan/Fereidan	2008	Autumn	2
116–130	Potato	Fars/Shiraz	2009	Summer	2
131–144	Potato	Hormozgan/Bandarabas	2009	Summer	2
145–146	Tomato	Khoozestan/Shushtar	2004	Summer	N2
147–150	Potato	Khoozestan/Andimeshk	2004	Summer	N2
151–152	Potato	Khoozestan/Dezful	2008	Summer	N2
153–154[a]	Potato	Khoozestan/Shushtar	2004	Summer	N2
155–170	Potato	Fars/Dehbid	2008	Autumn	N2
G1–G2[b]	Potato	Golestan/Gorgan	2008	–	2

[a] Reference biovar N2 isolates: 153 = Rs39, 154 = Rs38 (Nouri *et al.,* 2008).
[b] Reference biovar 2 isolates: G1–G2 (Moslemkhany *et al*., 2008).

Hayward (1964). To determine biovar affiliation, we evaluated the utilization of cellobiose, lactose, maltose, manitol, dulcitol, sorbitol, meso-inositol, L-tryptophan, D-trehalose, M-tatrate, L-tartrate, D-ribose, and D-xylose (Schaad *et al*., 2001).

Pathogenicity

Tomato seedlings (*Lycopersicon esculentum* cv. Early Orbano) were transplanted into pots and grown in greenhouses under natural light. Thirty seven representative isolates of biovar 2 (3, 4, 6, 10, 41, 50, 81, 82, 85, 86, 61, 107, 115, 110–112, 120, 123–125, 142, 130–135, 100–104, 14, 70 and G1–G2) and 26 isolates of biovar N2 were used for the inoculation. A suspension of each isolate was made in sterile distilled water and adjusted to 10^8 cfu mL. Plants at the fourth to fifth true-leaf stage were each inoculated with 1 mL of bacterial suspension into the stem using a sterile syringe with needle. Control plants were injected with sterile distilled water. Each isolate was tested in three plants and symptoms were monitored over 3 weeks. *Ralstonia solanacearum* was recovered from wilted plants inoculated with each of the isolates by streaking a loopful of extracts from stem sections in water, onto sucrose peptone agar (SPA) (Schaad *et al*., 2001).

Identification of *Ralstonia solanacearum* by PCR

Genomic DNA from *R. solanacearum* was extracted following the method of Weller *et al*. (2000). The specific primer pairs 630/631, DIV2F/R and DIV1F/R were used in the PCR assays (Fegan *et al*., 1998; Seal *et al*., 1999). The PCR reaction was carried out in 25 µL containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 200 µM each of dNTPs, 100 ng of primers, 1 U of *Taq* polymerase (Metabion, Martinsried, Germany) and 1 µL of DNA template (25 ng μL^{-1}). The PCR was performed in a Palm-cycler™ (Germany). Thermal profile consisted of: 94°C for 4 min for initial denaturation, 30 cycles of 94°C for 1 min, 60°C (primer pair 630/631) or 62°C (primer pairs DIV2F/R and DIV1F/R) for 1 min and 72°C for 2 min, followed by a final elongation step of 72°C for 10 min. The PCR products were analyzed on 1% agarose gels in TAE buffer and visualized by staining with ethidium bromide (Sambrook *et al*., 1989).

Phylotype determination

To determine the phylotype of all *R. solanacearum* isolates, four forward (phylotype-specific primers) and one reverse primer and *R. solanacearum* species-specific primers 759 and 760 were used in phylotype-specific multiplex PCR (pmx-PCR) (Fegan and Prior, 2005). Pmx-PCR was carried out in a 25 µL reaction mixture, containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 250 µM each of dNTPs, 50 mM KCl, 1.25 U of *Taq* polymerase (Metabion), 6 pmoles of primers Nmult21: 1F, Nmult21: 2F, Nmult23: AF, Nmult22: InF, 18 pmoles of primer Nmult21: RR, 4 pmoles of primers 759 and 760 and 1.5 µL of DNA template (25 ng μL^{-1}). The amplifications were performed in a Palm-cycler™ (Germany). After an initial denaturation of 5 min at 95°C, the DNA was amplified for 30 cycles including, 94°C (1 min), 59ºC (30 s) 72ºC (30 s), followed by a final extension cycle at 72ºC for 10 min. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer and visualized by staining with ethidium bromide (Sambrook *et al*., 1989). The phylotype of each isolate was determined according to the size of the amplified DNA (Fegan and Prior, 2005).

Rep-PCR amplification

The repetitive PCR (rep-PCR) method with ERIC, BOX and REP primers described by Louws *et al*., (1995) was assessed on 35 representative isolates of biovar 2 and 26 isolates of biovar N2. The ERIC, BOX, and REP primer sets were synthesized by Metabion. The rep-PCR amplifications were performed as described previously (Louws *et al*., 1994; Versalovic *et al*., 1994) in 25 µL volumes containing 200 µM of each dNTPs, 2 mM $MgCl_2$, 1.5 pmoles primers, 1.25 U of *Taq* polymerase and 2 µL of DNA template (25 ng μL^{-1}). The thermal profile consisted of an initial denaturation step (95°C, 2 min) followed by 35 cycles of 1 min at 94°C, 1 min at 52ºC (ERIC), 53ºC (BOX) or 44ºC (REP), and 8 min at 65°C, and a final extension cycle at 65ºC for 10 min (Louws *et al*., 1994). The PCR products were separated on 1.5% agarose gel electrophoresis in TAE buffer and visualized by staining with ethidium bromide (Sambrook *et al*., 1989). The PCR amplifications using the three primer sets were repeated three times to test the reproducibility of these assays.

Data analysis

The amplified fragments of each isolate were detected, using the Total Lab (v.1.1) program and were scored as 1 (present) or 0 (absent) and pairwise comparisons were made using NTSYSPC software (Exeter Software, New York) (Rademarker *et al*., 1998). Genetic relationships among and within the *R. solanacearum* isolates were determined by cluster analysis performed by UPGMA on distance matrices calculated with the Jaccard's coefficient (Rohlf, 2000).

Results

Physiological and biochemical tests

All isolates were gram-negative, aerobic and positive for catalase, oxidase, Kovac's, Tween 80 hydrolysis, starch hydrolysis, were able to produce nitrite from nitrate and were negative for gelatin liquefaction. They produced typically fluid, irregular white colonies with reddish-pink centers on TZC medium, with the exception of 26 isolates (from 145 to 170), which produced relatively small colonies that were less fluid than colonies of the other isolates. All isolates produced a brown pigment from tyrosine after 72 h, with the exception of the previously mentioned 26 isolates (145–170). The isolates utilized lactose, maltose, D-ribose and cellobiose, but not mannitol, sorbitol and dulcitol.

Based of phenotypic results, all isolates belonged to *R. solanacearum*. Isolates 145–170, utilized D-trehalose and L-tryptophan, while all other isolates did not, also their pectolytic activity was less than the other isolates. These isolates were recognized as biovar N2 (2T). All 170 isolates were unable to utilize Meso-inositol, L- tartrate and M-tartrate.

Pathogenicity determination

Tomato plants inoculated with biovar 2 isolates, showed wilting 10 to 14 days after inoculation. Plants inoculated with biovar N2 isolates showed wilting symptoms 20 to 23 days after inoculation. Isolates of biovar 2 were more aggressive than biovar N2 isolates. *Ralstonia solanacearum* was recovered from wilted plants inoculated with each of the isolates of *R. solanacearum*.

Specific PCR for identification

All assayed isolates produced an approximately 1 kb product in PCR reactions with the DIV2F/R primer pair and no PCR product with the DIV1F/R primer pair, supporting that the isolates belong to *R. solanacearum* division 2. Using the 630/631 primer pair all isolates produced a 320-bp amplification product, with the exception of 26 isolates (145–170), which confirmed that the 630/631 primer pair is specific for the detection of *R. solanacearum* race 3.

Phylotype of Iranian potato and tomato isolates

Based on multiplex-PCR analysis, the Iranian potato and tomato isolates belonged to phylotype II. Phylotype II isolates comprised biovars 2 and N2 isolates (Figure 2).

Fingerprint analysis of Iranian isolates based on the rep-PCR method

Reproducible DNA fingerprints were generated from genomic DNA of all tested *R. solanacearum* isolates. The rep-PCR DNA fingerprint, ranging in size from 250 bp-3.5 kb for Box, 150 bp-2 kb for Eric and 250 bp-3 kb for Rep primers were amplified. The data were analyzed by combining each fingerprint and revealed four groups (1–4), corresponding to different haplotypes, that were grouped in two main clusters (I and II) (Figure 3). The clusters I and II included, respectively, all biovar 2 (haplotypes 1 and 2) and N2 (haplotypes 3 and 4). Haplotype 1 comprised isolates 130, 125, 82, 85, 86, 129, 107. Haplotype 2 comprised isolates 3, 4, 8–10, 14, 15, 41, 50, 61, 70, 81, 100–103, 110–113, 120, 123, 131, 132, 134, 135, 142 and G1. Haplotype 3 included all biovar N2 isolates from potato. Haplotype 4 comprised biovar N2 isolates from tomato. The two main clusters were distinguished at a mean level of similarity of approximately 57%. The average similarity between isolates was 91% in the cluster I and 94% in the cluster II.

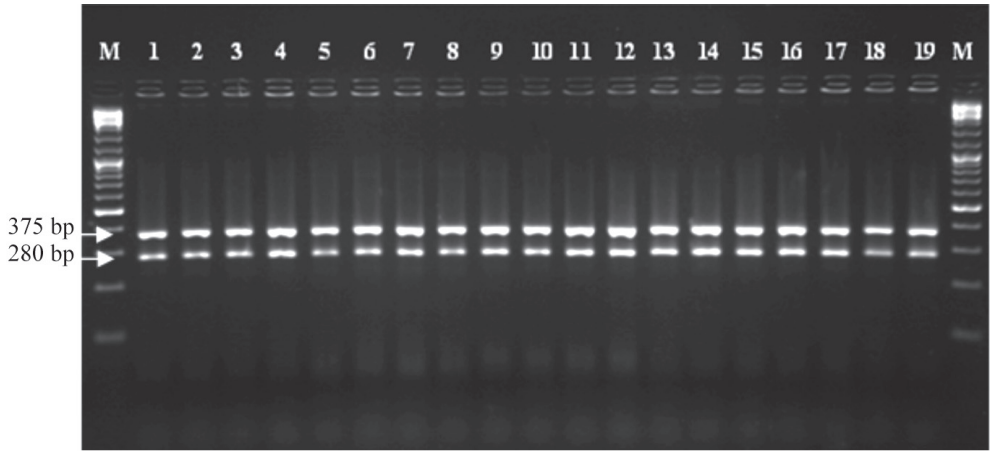

Figure 2. Agarose gel electrophoresis of pmx–PCR products from *Ralstonia solanacerum* isolates. Lanes: M, DNA molecular marker (Fermentas, cat no. SM 0331); 1, 169; 2, 145; 3, 170; 4, 155; 5, 146; 6, 160; 7, 165; 8, 154; 9, 148; 10, 90; 11, 40; 12, 1; 13, 16; 14, 70; 15, 10; 16, 120; 17, 11; 18, 81; 19, 100.

Discussion

Based on the phenotypic and pathogenicity characteristics, two distinct groups of *R. solanacearum* isolates were obtained from various geographic regions of Iran. The first group comprised 146 isolates of biovar 2/ race 3; the second group contained 26 biovar N2 isolates which were obtained from potato and tomato (Table 1). The Iranian biovar N2 isolates differed from the canonical phenotypic characteristics of biovar N2 in some aspects (failed to utilize Meso-Inositol, L-Tartrate, M-Tartrate and a lower pectolytic activity than biovar 2), but the other phenotypic characteristics were the same. By artificial inoculation, biovar 2 isolates were more aggressive than biovar N2 isolates on tomato plants. Our results indicate that pathogenicity tests on tomato can distinguish biovar 2 isolates from biovar N2 isolates. *Ralstonia solanacearum* has been found in Iran since 2001 (Bagheri and Taghavi, 2001; Maghooli *et al*., 2004; Irandoost *et al*., 2007; Nouri *et al*., 2008), but little is known about its distribution and diversity in various geographic regions. As indicated in the distribution of *R. solanacearum* in Iran shown in Figure 1, biovar 2 isolates are common and widely distributed, while biovar N2 is found only in some limited areas. In this study, ten biovar N2 isolates (Khozestan province) and 15 biovar N2 isolates (northern part of Fars province) have been isolated from warm and cool regions, respectively (Table 1); these isolates did not produce a PCR amplification product with the race 3 specific primers (630/631 primers). Biovar N2 isolates are often described as limited to warm climate zones (French *et al*., 1993; Marin and El-Nashaar, 1993; Horita *et al*., 2005; Nouri *et al*., 2008). Our findings suggest that Iranian biovar N2 isolates are able to adapt to cool-temperature zones. These results are relevant for epidemiology, because potato seed tubers are typically stored at 4 to 5°C for some months, and latently infected seed tubers are a common source of potato brown rot outbreaks.

The comparison of genomic fingerprint patterns of the Iranian isolates obtained by rep-PCR

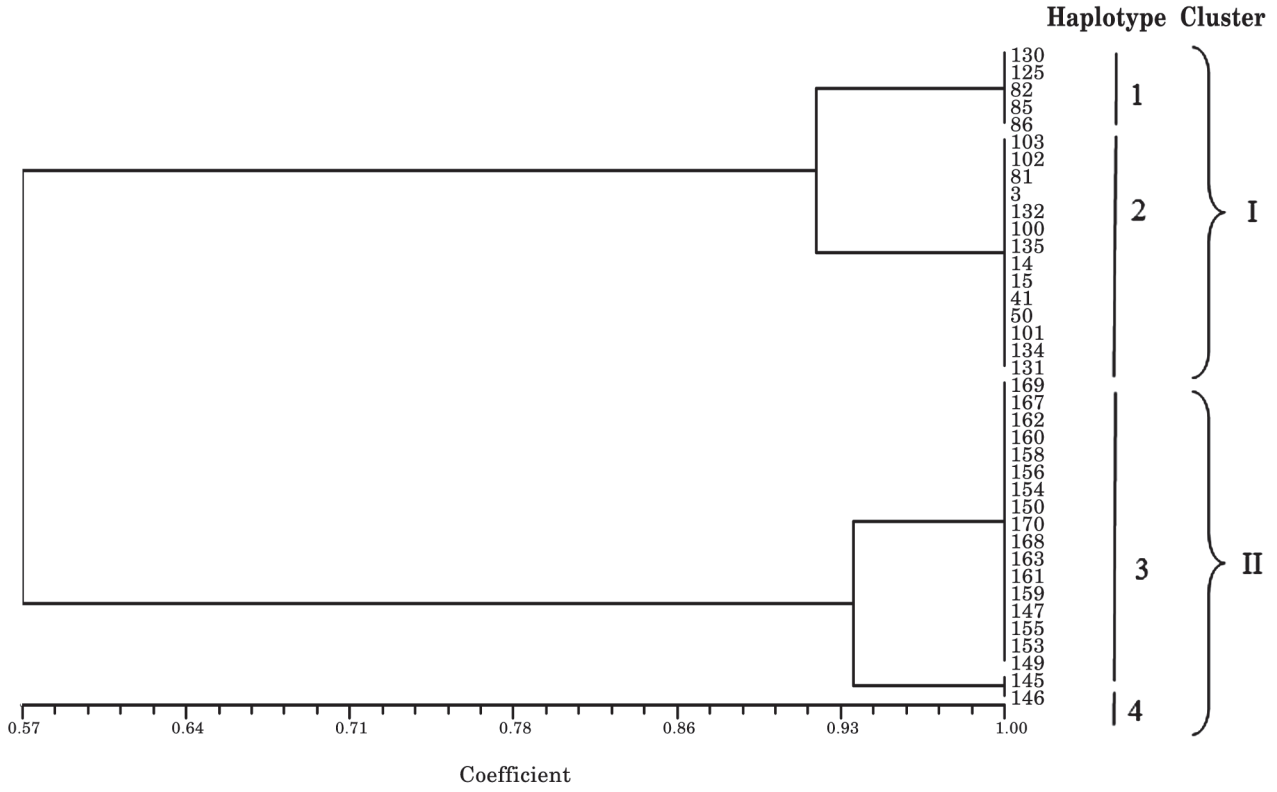

Figure 3. Dendrogram based on rep-PCR of *Ralstonia solanacearum* isolates with REP, ERIC and BOX primers.

revealed that the *R. solanacearum* biovar N2 and 2 isolates formed two distinct clusters, each divided in two groups (Figure 3). Our results showed a genetic homogeneity among biovar 2/ race 3 *R. solanacearum* isolates, confirming that biovar 2/ race 3 has relatively low levels of genetic diversity. Comparison of biovar 2/ race 3 populations isolated from potato pointed to a high level of genetic diversity in South America, while Kenyan populations consisted of only one major clone (Smith *et al*., 1995; 1998). Isolates of biovar 2/ race 3 appear to be more genetically variable in the region of origin of potato than in other parts of the world (Smith *et al*., 1995; 1998; Jeong *et al*., 2007; Nouri *et al*., 2008; Xu *et al*., 2009). Based on rep-PCR fingerprints, biovar N2 isolates formed two distinct groups that differ in original hosts (potato and tomato), suggesting that rep-PCR is a useful method for assessing the genetic relationships among biovars and hosts of *R. solanacearum* isolates (Horita and Tsuchiya, 2000; Norman *et al*., 2009). The clustering based on rep-PCR was confirmed by phenotypic features.

The phylotype determination is a new hierarchical classification: it is rapid and linked to geographic origin of species complex members (Fegan and Prior, 2005). Biovar N2 has several origins and it belongs to the three phylotypes II, III and IV. Phylotype III isolates belong to biovars 1, 2 and N2 from America (Fegan and Prior, 2005; Xu *et al*., 2009; Horita *et al*., 2010). Our results with pmx-PCR, as well as with specific primer pairs DIV2F/R and DIV1F/R, indicate that Iranian biovar N2 and 2 isolates belong to phylotype II/ division 2. Contrary to the majority of most Asian biovar N2 isolates, Iranian biovar N2 and 2 isolates seem to originate from South America. This finding confirmed previous reports in which the partial endoglucanase gene (*egl*) sequence of isolate Rs38 and Rs39 (EF647742 and EF647743) shared 100% sequence identity to the *egl* gene sequence (UW477) of the biovar N2 isolates from South American (Nouri *et al*., 2008).

Our results show genetic and phenotypic homogeneity among Iranian biovar N2 isolates and indicate that bacterial wilt of potato has been probably spread locally and internationally on latently infected potato tubers (Hayward, 1991). Therefore, we can speculate that biovar N2 was first introduced on potato seed tubers to Khoozestan, and afterwards moved to Fars province via infected tubers. Biovar 2/ race 3 probably originated in the Andes with potato, and isolates from around the world are nearly genetically and phenotypically identical, suggesting its distribution from South America by potato tubers (Hayward, 1994; Smith *et al*., 1995; Fegan and Prior, 2005; Xu *et al*., 2009). Biovar 2 and N2 isolates have probably been introduced to Iran by infected potato tubers. In the past, Iran imported seed potato tubers mainly from the Netherlands and Germany (Nouri *et al*., 2008), but, it is proper to mention that Iran has also imported potato tubers from South America in recent years.

The control of bacterial wilt is difficult; the best management strategies are planting disease-resistant hosts and preventing the introduction of latently infected potato tubers to new regions. Distribution and diversity maps will provide useful information to limit the introduction of *R. solanacearum* into new regions or hosts plants. To our knowledge, this is the first report of phylotype determination biovars of *R. solanacearum* with pmx-PCR in Iran.

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