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Research Article

The phenotypic and genotypic characteristics of causal agents of potato bacterial soft rot in Ardabil province of Iran

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Abstract: Bacterial soft rot causes severe damage to potatoes and is responsible for considerable economic losses in potato crop during both growing season and storage period. The most common causal agent of potato soft rot worldwide is *Pectobacterium carotovorum* (Pc); however in Iran, two other species: *P. atrosepticum* and *P. wasabiae* have been reported. Identification and assessment of genetic variation in pathogen populations are very important to understanding taxonomy, epidemiology, and management of a pathogen. In this study, potato stems and tubers showing soft rot symptoms along with their surrounding soil were collected from fields and seed storages of Ardabil province. A total of 33 pectolytic bacterial strains were isolated on nutrient agar and eosin methylene blue culture media. The isolated strains and five standard strains belonging to *P. atrosepticum, P. carotovorum* and *Dickeya dianthicola* were studied. The strains were identified as *P. carotovorum* subsp. *carotovorum* on the basis of phenotypic characteristics including: gram-negative, facultative anaerobic, soft rot production on potato slices, growth at 37 °C, and inability to produce acid from α-methyl-D-glucoside and also molecular identification using species-specific primers. Based on Y1/Y2 and ExpccF/ExpccR primers, the expected amplicons (434 and 550 bp fragments, respectively) were obtained for all strains and the standard strains belonging to *P. carotovorum.* According to rep-PCR and cluster analysis using UPGMA and NTSYS 2.1 software, the selected strains were categorized into two main groups and four subgroups. Rep-PCR indicated different levels of genetic heterogeneity among Pcc strains, however, no clear correlation was found between clustering and the geographical origin of the strains.

Keyword: *Pectobacterium carotovorum*, rep-PCR, species-specific primers

Introduction

Iran produces 5.4 million tons of potatoes annually, and stands as the 12th largest potato producer in the world as well as the fourth largest in Asia, after China, India and Bangladesh (FAO, 2013). Potatoes are cultivated mostly under irrigation in nearly all provinces of Iran. However, three major potato growing regions are the southern shores of the Caspian Sea (the Elburz Mountains), the Zagros Mountains, and the southern lowlands (CIP, 2012). One of the production centers in the Elburz is Ardabil province located in northwest of the country and it ranks second in potato production across Iran.

Bacterial soft rots have significantly limited

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potato production in the world. The main characteristic of this group of bacteria is the production of large amounts of pectolytic enzymes that induce rotting of plant tissues (Barras *et al*., 1994).

Soft rot coliforms belong to *Enterobacteriaceae*, *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pectobacterium atrosepticum* (Pba) (Gardan *et al.*, 2003), *Pectobacterium carotovorum* subsp. *brasiliensis* (Pcb)(Duarte *et al*., 2004), *Pectobacterium wasabiae* (Pwa) (Baghaee-Ravari *et al*., 2011, Pitman *et al*., 2008), *P. carotovorum* subsp. *odoriferum* (Pco) (Waleron *et al.*, 2014) and *P. betavasculorum* (Pbt) (Nabhan *et al.*, 2012) along with several *Dickeya* spp. including *D. dianthicola*, *D. dadantii*, *D. zeae*, *D. solani* (Toth *et al.*, 2011; van der Wolf *et al.*, 2013). These bacteria can cause soft rots in potatoes and consequently heavy economic losses in many commercial crops both in the field and during storage (Czajkowski *et al.*, 2015). Among them, Pcc is the most geographically diverse and has the widest host range (Charkowski, 2006; Toth *et al*., 2003). Different methods are employed to detect, identify and differentiate between pectinolytic *Pectobacterium* and *Dickeya* species using selective growth agar media, biochemical and physiological tests (Schaad *et al*., 2001), whole-cell fatty acid analysis (Dawyndt *et al.*, 2006), serological (Gorris *et al.*, 1994) and molecular techniques. Traditional methods are time-consuming and swayed by low sensitivity and specificity.Therefore PCR-based detection methods are used routinely to identify *Pectobacterium* spp. given their high specificity and rapid detection. The most common PCR-based detection methods include 16S rDNA sequence analysis (Kwon *et al.*, 1997), ITS-PCR (Pitman *et al.*, 2008; Toth *et al.*, 2001), repetitive sequence-based PCR (rep-PCR) (Baghaee Ravari *et al*., 2013; Rezaei and Taghavi, 2010; Versalovic *et al.*, 1991), restriction fragment length polymorphism (RFLP) (Darrasse *et al.* 1994; Gardan *et al.*, 2003; Rahmanifar *et al*., 2012), amplified fragment length polymorphism (AFLP) (Avrova *et al.*, 2002), multi locus sequence tagging (MLST) (Nabhan *et al.*, 2012) and random amplification of polymorphic DNA (RAPD) (Mäki-Valkama and Karjalainen, 1994). In this study, phenotypic methods and PCR-based detection in combination with repetitive sequence-based PCR, the BOX-AIR primer (Treangen *et al.*, 2009) were employed for identification and phylogenetic analysis of *Pectobacterium* spp. obtained from infected potato plants in Ardabil province. The species-specific primers used included Eca1f/Eca2r, specific for Pba (De Boer and Ward, 1995), EXPCCF/ EXPCCR, specific for Pcc and Pwa strains (Kang *et al.*, 2003), and Y1/Y2, specific for *Pectobacterium* spp. except for *P. betavasculorum* and *Dickeya* (Darrasse *et al.*, 1994).

Materials and Methods

Sampling and strain collection

Suspected strains of *Pectobacterium* spp. were selected from the margin of infected potato stem and tuber samples which were collected from different fields and seed storages in Ardabil Province. Bacterial cultures were performed on nutrient agar (NA) and eosin methylene blue agar (EMB-agar) media. After incubation at 24 ℃ for 48-72 h, single colonies with a milky white and green metallic color were subcultured on NA medium. All strains were stored in the nutrient broth medium containing 25% (v/v) glycerol at -20 ºC for further studies (Schaad *et al*., 2001). Reference strains used in this study were *Pectobacterium atrosepticum* SCRI1043 provided by Dr. Minna Pirhonen (Helsinki University, Finland) and *Dickeya dianthicola* 2114, *P. atrosepticum* 1007, *P. carotovorum* 1955 and 1949 provided by Patricia van der Zouwen (Wageningen Plant Research Institute, Netherlands).

Biochemical and physiological tests

Biochemical tests conducted in the present study were gram reaction, potato soft rot, oxidation/fermentation of glucose, oxidase and catalase reactions, fluorescent pigment on King's B medium, production of reducing substances from sucrose, phosphatase activity, sensitivity to erythromycin with 15µg per disk, indole production, starch, gelatin and esculin hydrolysis, lecithinase (Fahy and Hayward,

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1983), H_2S production from cystein, arginine dihydrolase, nitrate reduction, utilization of citrate, D-tartrate and malonate, acid production from α-methyl-D-glucoside, trehalose, sorbitol, arabitol, arabinose, inulin, melibiose, raffinose, mannitol and lactose (Schaad *et al.*, 2001).

Preparation of Bacterial DNA

DNA extraction was performed using the whole cell alkaline lysis method (Rademaker and de Bruijn, 1997). Extracted DNA was analyzed by electrophoresis on a 1% agarose gel and stored at 4 ºC or -20 °C until used.

Molecular detection by specific primers

Polymerase chain reaction (PCR) was performed using primer pair Y1 (5′-TTACCGGACGCC GAGCTGTGGCGT-3′) and Y2 (5′-CAG GAAG ATGTCGTTATCGCGAGT-3′) (Yahiaoui *et al*., 2003) in 25µL of a reaction mixture containing 2.5µL of PCR buffer 10X, 0.75µL $MgCl_2$ (25mM), 0.3µL dNTPs (10mM), 0.5µL of each primer (10 pmol/µl), 0.3µL of Taq DNA polymerase (5 u/µl) and 2µL of the DNA extract. PCR amplification was performed using a Thermocycler (Bio-rad, MJ Mini, USA) using the following program; initial denaturation for 5 min at 94 °C, 34 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min.

Also, PCR was carried out using ExpccF (5′-GAACTTCGCACCGCCGACCTTCTA-3′) and ExpccR (5′-GCCGTAATTGCCTACCTGCTT AAG-3′) primers under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min (Kang *et al*., 2003). The primers Eca1F (5′-CGGCATCATAAAAACACG-3′) and Eca2R (5′-GCACACTTCATCCAGCGA-3′) (De Boer and Ward, 1995) were used under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 62 °C for 45 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 8 min. Amplification products were analyzed by electrophoresis on 1% agarose gels.

Repetitive Sequence-based PCR

BOX-PCR was executed using the BOX A1R primer (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al*., 1991) under the following conditions: each reaction (25µL) containing 2.5µL of PCR buffer 10X, 1.5µL $MgCl_2$ (50mM), 0.5µL DMSO 100%, 16µL ddH_2O, 1.25µL mix of dNTP's 10mM, 0.6µL primer (50 pmol BOX-AIR), 0.4µL of Taq DNA polymerase (5u/µl). PCR consisted of an initial denaturation at 95 °C for 7min, followed by 30-35 cycles of denaturing at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 65 °C for 6min, and a final extension at 65 °C for 16 min. Cluster analysis was conducted by UPGMA method (unweighted pair-group method, Jaccard coefficient) using NTSYS-pc 2.1 software.

Results

Biochemical and physiological tests

Out of 60 strains isolated, biochemical and physiological tests were performed on 33 pectolytic bacterial strains and five reference strains (Table 1). All the isolated strains were identified as *P. carotovorum*. Strains were gram-negative, able to produce soft rot on potato slices, aerobic/anaerobic growth, oxidase negative, catalase positive, non-fluorescent on King´s B (KB) medium. Starch hydrolysis and acid production from arabinose, inulin and sorbitol were negative, whereas gelatine and esculin hydrolysis were positive. Other characteristics are listed in Table 1. The strains exhibited similarity in physiological and biochemical tests. However, some had variations in a few characteristics such as lecithinase, phosphatase activity and utilization of a few carbon sources. All strains grew at 37 °C and were resistant to erythromycin. Most of the strains did not produce acid from alpha-methyl-D-glucoside. These findings support the identification of isolated strains as *P. carotovorum* rather than *P. atrosepticum*, *P. wasabiae* and *Dickeya* spp.

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Table 1 Phenotypic characteristics for identification of causal agents of potato soft rot isolated in this study (obtained from Ardabil province, 2013) compared to reference strains.

Tests	Pba	Pc	Dd	Positive strains (%)
Oxidation/fermentation of glucose	F/+	F/+	F/+	100
Potato soft rot	+	+	+	100
Blue pigment on PDA	-	-	-	0
Growth in 5% NaCl	+	+	+	100
Growth at 37 °C	-	+	+	100
Production of reducing substances from sucrose	-	-	-	0
Nitrate reduction	+	+	+	100
H_2S production from cysteine	+	+	+	100
Lecithinase	-	-	+	66.6
Indole production	-	-	-	0
Arginine dihydrolase	-	-	-	
Sensitivity to erythromycin	-	-	+	0
Phosphatase activity	-	-	-	6.1
Acid production from				
Alpha-methyl-D-glucoside	+	-	-	12.0
Trehalose	+	+	+	100
Arabinose	-	-	+	100
Melibiose	+	+	+	100
Raffinose	+	+	+	100
Mannitol	+	+	+	100
Lactose	+	+	+	85.0
Utilization of:				
D-tartrate	-	-	-	18.1
Malonate	-	-	-	9.1
Citrate	+	+	+	100

+: positive strains; -: negative strains; Pba: *Pectobacterium atrosepticum*; Pc: *Pectobacterium carotovorum*; Dd: *Dickeya dianthicola*.

Cluster analysis based on the results of phenotypic features and strain grouping were performed using UPGMA method (Fig. 1). The strains were divided into four distinct groups. The similarity coefficients among strains ranged from 0.75 to 1.

Molecular detection by specific primers

PCR using species-specific primers (Y1/Y2, Eca1F/Eca2R and ExpccF/R) was used to determine whether *Pectobacterium* spp. or *Dickeya* spp.were present in individual plant samples. The expected amplicons totaling about 434 and 550 bp were obtained using Y1/Y2 and ExpccF/R primers for all isolated and standard strains belonging to *P. carotovorum*. The results obtained from some strains are shown in Figures 2 and 3. Also, a 690 bp fragment was only amplified using Eca1F/Eca2R primers for *P. atrosepticum* strains 1007 and SCRI1043.

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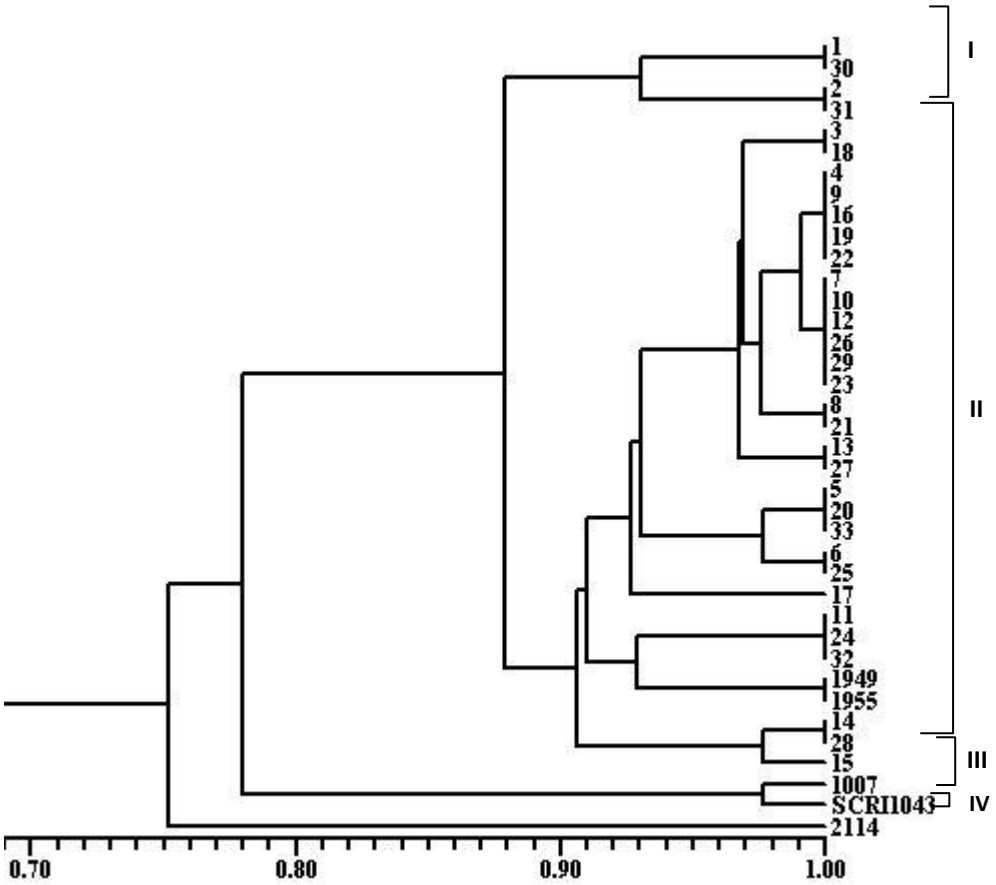

Figure 1 Cluster analysis of isolated strains based on the results of phenotypic data (+ and -) by NTSYS 2.1 software using UPGMA method (unweighted pair-group method, using simple matching coefficient). 2114: *D. dianthicola*; 1007: *P. atrosepticum*; SCRI1043: *P. atrosepticum*; 1955: *P. carotovorum*; 1949: *P. carotovorum*

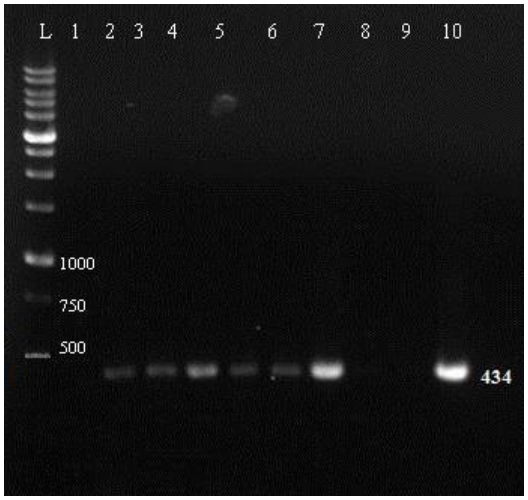

Figure 2 PCR products resulting from the amplification of 434 bp fragment using Y1 / Y2 primers on 1.2% agarose gel. L: 100bp DNA Ladder plus Bioron; 1: distilled water; 2,3,4,5, 6 and 7: selected strains; 8: *D. dianthicola* 2114; 9: *D. dianthicola* 2114; 10: *P. carotovorum* 1955.

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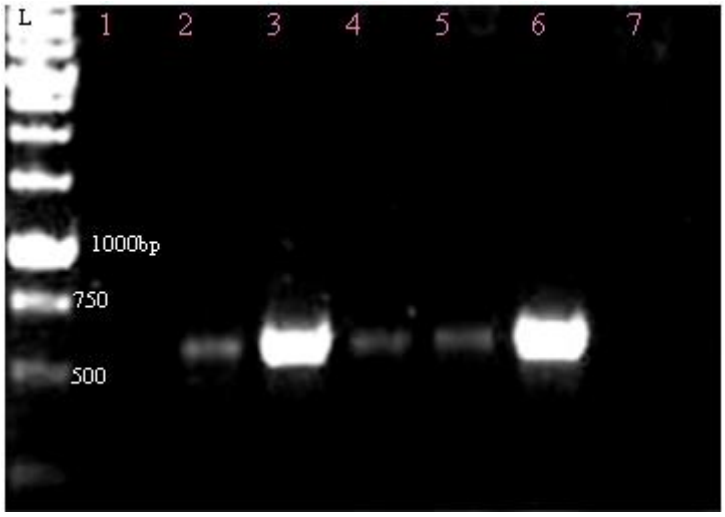

Figure 3 PCR products resulting from the amplification of 550 bp fragment using ExpccF/R primers on 1.2% agarose gel. L: 100bp DNA ladder plus Bioron; 1: distilled water; 2, 3, 4 and 5: selected strains; 6: *P. carotovorum* 1955; 7: *D. dianthicola* 2114.

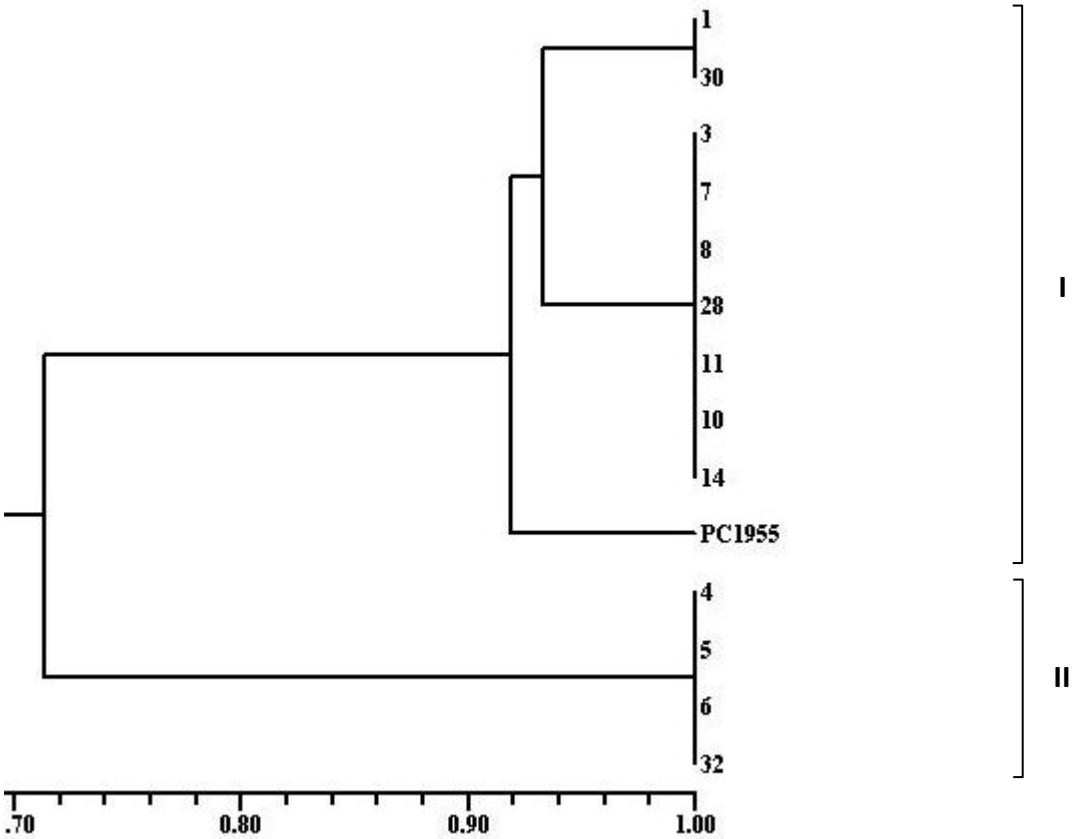

Figure 4 Cluster analysis of selected strains of *P. carotovorum* based on the results of rep-PCR (using BOX-AIR primer by NTSYS 2.1 software and UPGMA method (unweighted pair-group method, using Jaccard's coefficient).

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Rep-PCR genomic fingerprinting

To study genetic diversity, 13 representative strains of Pcc were selected, and interspersed repetitive DNA sequences in their genomes were investigated by rep-PCR, using BOX-AIR primer (Treangen *et al.*, 2009). The number and size of DNA fragments obtained in genomic fingerprinting were 11-13 bands, ranging from 150 to 3000 bp. Building on UPGMA method, the results revealed two main groups of strains with similarity value of approximately 72%. The first group was divided into three subgroups at 92% similarity where subgroup III was related to Pc 1955 standard strain. The second group showed one genotype (Fig. 4).

Discussion

In this research, we identified the pectolytic bacterial strains isolated from potato plants in Ardabil province using a combination of biochemical and molecular tests in order to accurately identify the relevant causal agent. The genetic diversity of the strains was determined by the rep-PCR technique. Based on biochemical tests, previous studies suggested that *P. atrosepticum* and *P. wasabiae* do not grow at 37ºC and that *P. atrosepticum* and *P. betavasculorum* are positive in the α-methyl glucoside test (Hauben *et al*., 1998; Gardan *et al*., 2003). In the present study, resistance of the strains to erythromycin, growth of all strains at 37 °C, and negative reaction of most of the strains (88%) in the α-methyl glucoside test support the identification of isolated strains as *P. carotovorum* rather than the closely related pectolytic *P. atrosepticum, P. wasabiae* and *Dickeya* spp.

Phenotypic discrimination using the traditional bacteriological methods is challenged due to various phenotypic characteristics among the strains of a species. Therefore, accurate identification solely based on biochemical tests has become more difficult. Studies have also shown that physiological and biochemical methods cannot clearly distinguish between related members of the *Pectobacterium* spp. (Pitman *et al*., 2008).

Molecular diagnostic techniques such as species-specific primers, ITS-PCR and PCR-RFLP have provided easy and rapid identification of bacterial strains (Toth *et al*., 2001). In this project, PCR was conducted using specific primers to the pectate lyase (*pel*) gene (Y1/Y2), which are able to produce a 434 bp fragment in all *Pectobacterium* spp. except for Pbt and *Dickeya* (Yahiaoui *et al*., 2003). Moreover, ExpccF/R primers were used to amplify a 550 bp fragment for Pcc and Pwa strains. According to previous studies, 434 and 550 bp DNA fragments were produced by all strains except for *Dickeya dianthicola* and Pba standard strains (Baghaee-Ravari *et al*., 2011; Kang *et al.*, 2003; Yahiaoui *et al*., 2003). Isolated strains were screened using Eca1F/Eca2R primers (De Boer and Ward, 1995), and unlike some reports from Iran, they failed to amplify with Pba specific primers (Baghaee Ravari *et al*., 2011; Tavasoli *et al*., 2011).

Therefore, biochemical characterizations and species-specific primers differentiated the studied strains from different *Pectobacterium* and *Dickeya* species and identified them as Pcc. These subspecies were found to be the most important causal agent of soft rot disease of potatoes in Ardabil province. Some studies show that Pcc is the most prevalent soft rot bacteria of potatoes in Iran and worldwide (Amdan *et al*., 2015; Baghaee Ravari *et al*., 2013; Czajkowski *et al*., 2015; De Boer *et al*., 2012; Firouz, *et al*., 2006; Rahmanifar *et al*., 2012; Rezaei and Taghavi, 2010; Serfontein *et al*., 1991; Yahiaoui-Zaidi *et al*., 2003).

Repetitive extragenic palindromic elements (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements have been developed to target the repetitive sequences present in bacterial genomes and are commonly known as repetitive sequence-based PCR (REP-PCR) (Versalovic *et al*., 1991). These three fingerprinting techniques provide the banding profiles from the bacterial genome that can be used in clustering the pathogen isolates from genus down to strain level (Czajkowski *et al.*, 2015). In the present study,

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rep-PCR using BOX primer was conducted to study genetic heterogeneity within Pcc strains. BOX elements are widespread in the genomes of different bacterial groups and contribute to structural dynamics of the bacterial genome (Treangen *et al*., 2009; Versalovic *et al*., 1991). Results showed that Pcc strains and Pc1955 were grouped into four clusters at 92% similarity coefficient. Amplification of the sequences between each of these repetitive elements indicated that the identified Pcc strains were genetically variable. Therefore, the Pcc strains are phenotypically and genetically heterogeneous (Avrova *et al*., 2002; Baghaee Ravari *et al*., 2013; Rezaei and Taghavi, 2010). Given that the strains from different locations, e.g., Arzan, Piraghom, Samian, Topraghlo, Khalil Abad and Seid Abad of Ardebil were grouped into one cluster, there was no relationship between clustering based on the rep-PCR and geographical origin of the strains. Wide host range and geographical distribution may have caused genetic diversity in this species (Avrova *et al*., 2002). The present findings also confirmed that the rep-PCR technique is of reliable discriminatory power in evaluating the diversity of Pcc strains.

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خصوصیات فنوتیپی و ژنوتیپی پکتوباکتری‌های عامل بیماری پوسیدگی نرم سیب‌زمینی در استان اردبیل

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چکیده: پوسیدگی نرم از بیماری‌های مهم باکتریایی سیب‌زمینی است که باعث خسارت شدید اقتصادی به محصول در مزرعه و انبار می‌شود. باکتری (Pc) *Pectobacterium carotovorum* معمول‌ترین عامل پوسیدگی نرم سیب‌زمینی است. اما در ایران *P. atrosepticum* و *P. wasabiae* (Pwa) نیز به‌عنوان عوامل پوسیدگی نرم باکتریایی گزارش شده‌اند. شناسایی و بررسی تنوع ژنتیکی در جمعیت عوامل بیماریزا از اهمیت زیادی در تاکسونومی، اپیدمیولوژی و مدیریت برخوردار است. در این بررسی از مزارع سیب‌زمینی و انبارهای مهم نگهداری غدد بذری در سطح استان اردبیل بازدید و از ساقه و غده‌های سیب‌زمینی با علایم پوسیدگی نرم به‌همراه خاک اطراف آنها نمونه‌برداری انجام شد. سپس ۳۳ جدایه پکتولیتیک روی محیط‌های کشت نوترینت آگار و ائوزین متیلن بلو آگار جداسازی شد و به‌همراه پنج جدایه استاندارد متعلق به گونه‌های *P. atrosepticum*، *P. carotovorum* و *Dickeya dianthicola* مورد بررسی قرار گرفت. جدایه‌ها براساس خصوصیات فنوتیپی شامل گرم منفی، بی‌هوازی اختیاری، تولید پوسیدگی نرم در ورقه‌های سیب‌زمینی، عدم استفاده از آلفا متیل گلوکوزید، رشد در ۳۷ درجه سانتی‌گراد و سایر آزمون‌ها و شناسایی مولکولی با استفاده از آغازگرهای اختصاصی گونه ExpccF/ExpccR، Y1/Y2 و Eca1F/Eca2R به‌عنوان *P. carotovorum* subsp. *carotovorum* شناسایی شدند. با پرایمرهای Y1/Y2 و ExpccF/ExpccR به‌ترتیب قطعات مورد انتظار ۴۳۴ و ۵۵۰ جفت بازی در تمام جدایه‌ها و هم‌چنین جدایه‌های استاندارد متعلق به *P. carotovorum* تکثیر شد. تنوع ژنتیکی در جدایه‌های منتخب به روش مولکولی rep-PCR با آغازگر BOX-AIR مورد بررسی قرار گرفت. براساس تجزیه خوشه‌ای داده‌ها به روش UPGMA و توسط نرم‌افزار NTSYS pc 2.1جدایه‌ها در دو گروه اصلی و چهار زیر گروه قرار گرفتند که نشان‌دهنده تنوع ژنتیکی در بین جدایه‌های مختلف بود ولی بین جدایه‌های هر گروه و منطقه جغرافیایی جدایه‌ها ارتباطی وجود نداشت.

واژگان کلیدی: آغازگرهای اختصاصی گونه، *Pectobacterium carotovorum*، rep-PCR

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