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Characterization of *Pectobacterium* species from Iran using biochemical and molecular methods

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Abstract Characteristics of forty strains from macerated potato tubers and water-soaked lesions of some ornamental plants were studied in north parts of Iran. The causal organisms isolated from infected tissues were identified as *Pectobacterium* spp. based on their physiological and biochemical assays and confirmed by species and subspecies specific PCR and RFLP analysis of 16S–23S intergenic transcribed spacer region. Artificial inoculation of isolates to their related hosts generated the same symptoms on potato and ornamental plants, from which the same bacteria were isolated and identified. We detected two groups of atypical isolates in this study. The first group from potato classified as *Pectobacterium carotovorum* subsp. *carotovorum* by phenotypic tests but was unable to elicit HR on tobacco leaves, to grow at 37°C and to amplify the *pel* gene relevant to this subspecies. The second one from ornamental plants which was again characterized as *Pectobacterium carotovorum* subsp. *carotovorum* in biochemical assays, produced a unique ITS-RFLP profile different from all of known *Pectobacterium* species and subspecies. Our findings based on phylogenetic analysis using concatenated partial sequences of housekeeping genes *mdh* and *gap*A, indicated the occurrence of *P. wasabiae* as a novel species in potato storage in Iran. Furthermore we detected a distinct clade of *Pectobacterium* spp. from some ornamental plants including *Schlumbergera bridgesii, Syngonium podophyllum* and *Iris* spp.

Keywords Housekeeping genes · Ornamental plants · Potato · *Pectobacterium* strains · Soft-rot · Plant disease

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Introduction

Pectobacterium spp. cause a spectrum of disease symptoms (termed wilt, soft rot, and blackleg) on a wide range of monocot and dicot host plants. These diseases are responsible for large economic losses in potato and ornamental plants. This genus has been subjected to extensive taxonomic research and consequently, divided into several species and subspecies on the basis of molecular, biochemical and host range

differences (Kwon et al. 1997; Hauben et al. 1998; Gardan et al. 2003). To date, five *Pectobacterium* species have been described: *P. atrosepticum*, *P. betavasculorum*, *P. carotovorum*, *P. wasabiae* (Gardan et al. 2003) and the recent one *P. brasiliensis* (Daurte et al. 2004; Van der Merwe et al. 2010), which is phylogenetically further apart from the other four *Pectobacterium* spp. (Ma et al. 2007).

The species *P. betavasculorum* and *P. atrosepticum* constitute an exception to the broad-host range nature of *Pectobacterium* spp, since they have been reported almost exclusively on sugar beet and potato respectively (Ma et al. 2007), whereas other potato-infecting species, *P. carotovorum*, *P. brasiliensis*, and *P. wasabiae* have been reported to cause disease on other plant species (Pitman et al. 2008; Kim et al. 2009).

P. carotovorum subsp. *carotovorum* has been previously isolated from potato producing areas in Iran (Soltani-Nejad et al. 2005; Firoz et al. 2007). *P. atrosepticum* also has been reported in south-west Iran, but the identification was based only on biochemical and physiological characteristics (Zohour Paralak et al. 2007). To this date, there is no report of other related species of *Pectobacterium* on potato from Iran.

The north-eastern region of Iran accounts for one of the largest areas of potato production. Postharvest losses in this crop due to diseases, inadequacy in packing and lack of refrigeration facilities is significant. Farmers in these areas keep their seed tubers in indoor stores annually and, therefore, tuber contamination with pectolytic bacteria is one of the main causes of postharvest losses in this crop. The occurrence of pectolytic enterobacteria causing soft rot and leaf spot of ornamental plants have been previously reported in many countries (Chao et al. 2006; Boyraz et al. 2006; Kim and Kim 2007; Alippi and Lopez 2009), including greenhouses of northern Iran (Khalemlou 2007).

In this research we evaluated the diversity of *Pectobacterium* spp. isolated from potato and ornamental plants in northern Iran. The objective of the present study was to confirm the identity of *Pectobacterium* spp. as the causal organisms of soft rot, stem rot and leaf spot through biochemical and molecular techniques. Our findings showed the presence of *P. wasabiea* as a destructive enterobacterium in the potato storages in north-east of Iran. We also detected a possible new species of *Pectobacterium* in ornamental plants.

Materials and methods

Bacterial strains

The bacterial strains used in this study were isolated from diseased potato tubers and water-soaked lesions of ornamental plants (Table 1). The type strains of *Pectobacterium carotovorum* subsp. *carotovorum* (IBSBF-863 = ATCC15713), *Pectobacterium atrosepticum* (IBSBF-1819 = ATCC33260), *Pectobacterium betavasculorum* (IBSBF-787 = ATCC43762), *Pectobacterium carotovorum* subsp. *odoriferum* (IBSBF-1814 = ICMP11533) *Dickeya chrysanthemi* bv. *chrysanthemi* (IBSBF-231 = ATCC11663) obtained from the Instituto Biológico Seção de Bacteriologia Fitopatologia (IBSBF) and reference strain of *Pectobacterium wasabiae* (SCRI 488) obtained from Scottish Crop Research Institute (SCRI) were included for comparison in different tests.

Media and cultural condition

Isolation of bacteria from diseased samples was carried out according to Perombelon and Van der Wolf (2002). Briefly, small amounts of leaf and tuber samples from the margin of healthy and diseased tissue were homogenized in 1-2 drops of sterile water, allowing 10 min to stand. The suspensions were plated on modified crystal violet pectate (CVP) medium (Perombelon and Hyman 1986) and incubated at room temperature to allow isolation of *P. atrosepticum*. Single colonies obtained on CVP medium were purified on King's B medium and selected strains were further characterized. All isolates were stored in sterile water at room temperature and in 30% (*v/v*) glycerol at −80°C.

Phenotypic tests

Isolates that were characterized as positive in pectolytic activity were submitted for the conventional biochemical and physiological tests including Gram reaction, fermentative metabolism (Hugh and Leifson 1953), oxidase and catalase activity. Hydrolysis of

Table 1 *Pectobacterium* strains isolated from potato and ornamental plants in northern Iran

Store/greenhouse	No. of strain(s)	Geographical origin in Iran	Host species	Plant part
S1	2(IR-Po1, IR-Po2)	Northern Khorasan- Ghochan	*Solanum tuberosum*	Tuber
S2	6(IR-Po3, IR-Po4, IR-Po8 to IR-Po11)	Northern Khorasan-Shirvan	*S. tuberosum*	Tuber
S3	3(IR-Po5 to IR-Po7)	Northern Khorasan- Ghochan	*S. tuberosum*	Tuber
S4	2(IR-Po12, IR-Po13)	Northern Khorasan-Bojnord	*S. tuberosum*	Tuber
S5	2(IR-PoG, IR-PoG2)	Northern Khorasan-Bojnord	*S. tuberosum*	Tuber
S6	3(IR-PoC3 to IR-PoC5)	Northern Khorasan-Bojnord	*S. tuberosum*	Tuber
G1	3(IR-Ph1, IR-Ph9,IR-Ph15)	Gilan-Chaboksar	*Philodendron scandens*	Leaf
G1	4(IR-AG17- IR-AG20)	Gilan-Chaboksar	*Aglaonema* sp.	Leaf
G2	1(IR-KA2)	Mazandaran-Amol	*Schlumbergera bridgesii*	Leaf
G3	4(IR-KA6 to IR-KA9)	Golestan-Kordkooy	*Schlumbergera bridgesii*	Leaf
G4	2(IR-Is2, IR-Is3)	Mazandaran-Sari	*Iris* sp.	Tuber
G5	3(IR-S4, IR-S6)	Gilan-Lahijan	*Syngonium podophyllum*	Leaf
G6	5(IR-S10, IR-S14)	Gilan-Lahijan	*Syngonium podophyllum*	Leaf

gelatin, lecithin and casein were tested on gelatin agar, on egg-yolk agar and on skimmed-milk agar, respectively (Dickey and Kelman 1988). Acid production from inulin in phenol red peptone water, production of reducing substances from sucrose, malonate utilization, indole production from tryptophan, anaerobic degradation of arginine (Moeller medium) and utilization of citrate (Simmons' medium) were studied using previously described methods (Gallois et al. 1992; Gardan et al. 2003). They also were checked for production of phosphatase, sensitivity to erythromycin (Schaad et al. 2001), ability to grow at 37°C in nutrient broth and growth in 5% sodium chloride on nutrient agar at 28°C. In addition, the assimilation of carbon sources were tested on the basal medium of Ayers et al. (1919) supplemented with 0.1% carbohydrates including trehalose, lactose, α-methyl glucoside, D-(+)-arabitol, D-(+)-melibiose, D-(-)-tartrate ,raffinose, sorbitol, maltose, cellubiose and palatinose.

Virulence assays

Potato Virulence assays were performed with potato stems and tubers (*Solanum tuberosum* cv. Marfana). For stem pathogenicity, tubers were planted in 12-cm diameter pots and grown in a greenhouse until the stems were 20–30 cm. Inoculation was carried out by injecting 10 µl of a 48 h culture of bacterial cells grown in Nutrient Agar (NA), into each stem 5 cm above the soil line and covered with parafilm. Three stems per plant and four replica plants per strain were tested. Inoculated plants were covered with plastic bags to maintain high humidity for 48 h in growth chamber at 24°C and evaluated for symptoms up to 21 days after inoculation (Duarte et al. 2004). Reisolations from inoculated plants were confirmed by pectolytic assay, morphology and biochemical tests. Statistical analysis of data was conducted using the Statistical Analysis Systems (SAS Institute, Cary, NC). Analysis of variance was done using the general linear model procedures.

Ornamental plants Inoculation was done by injecting 100 µl of bacterial suspension (10^7 CFU/ml) into the surface of ornamental leaves with a syringe and incubated them in moist chamber at 28°C for 48 h (Kim and Kim 2007). Two independent experiments were performed and 4 leaves per strain were inoculated. Positive *(*ATCC 15713, ATCC 33260 and SCRI 1043 strains) and negative (water) controls were included in these experiments.

An assay for the HR was performed essentially as described in Yap et al. (Yap et al. 2004). *Nicotiana tabacum* cv. Xanthi leaves were infiltrated with a suspension containing 2×10^8 CFU/ml. Plants were examined for HR elicitation after 24–48 h.

Bacterial DNA extraction

Total genomic DNA was extracted from 24-h cultures grown on NA using a commercial extraction kit as

suggested by the manufacturer (Accuprep DNA extraction kit- k3032, Bioneer, Korea) and stored at −20°C.

Molecular identification

Specific PCR assays were done with three pairs of primers (Table 2) to detect *Pectobacterium* strains as described below: primers EXPCCR and EXPCCF were performed to amplify a 550 bp band in *P. carotovorum* subsp. *carotovorum* (Kang et al. 2003). Primers ECA1f and ECA2r were used in PCR reactions as described by De Boer and Ward (1995) to detect *P. atrosepticum*. Also primers Y1 and Y2 selected from a pectate lyase-encoding gene of the Y family (Darrasse et al. 1994) were used to identify *P. carotovorum* subspecies. PCR was performed in 25 μl of a reaction mixture containing 2.5 μl of 10×PCR buffer, 2 mM $MgCl_2$, 200 μM of deoxynucleoside triphosphates, 0.8 μM of each primer, 0.5 U of Taq polymerase (Roche Diagnostics) and 50 ng of template DNA. PCR amplification was carried out using thermal cycler (Applied biosystems 2720, USA) with the following thermal regime: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles of 94°C for 30 s, 60°C (EXPCCR/ EXPCCF and Y1/Y2) or 67°C (ECA1f/ ECA2r) for 45 s, and 72°C for 1 min, ending with incubation at 72°C for 10 min. In all cases, amplified DNA fragments were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Table 2 Primers used in this study

Primer	Sequence (5′ to 3′)
L1	CAAGGCATCCACCGT
G1	GAAGTCGTAACAAGG
Y1	TTACCGGACGCCGAGCTGTGGCGT
Y2	CAGGAAGATGTCGTTATCGCGAGT
ECA1f	CGGCATCATAAAAACACG
ECA2r	GCACACTTCATCCAGCGA
EXPCCF	GAACTTCGCACCGCCGACCTTCTA
EXPCCR	GCCGTAATTGCCTACCTGCTTAAG
gapA326F	ATCTTCCTGACCGACGAAACTGC
gapA845R	ACGTCATCTTCGGTGTAACCCAG
mdh86F	CCCAGCTTCCTTCAGGTTCAGA
mdh628R	CTGCATTCTGAATACGTTTGGTCA

The 16S-23S intergenic transcribed spacer (ITS) PCR assay was also performed with general primers L1 and G1 (Table 2) for all strains according to Toth et al. (2001). Each 50 μl pre-amplification reaction contained 5 μl of 10×PCR buffer, 2 mM $MgCl_2$, 200 μM of deoxynucleoside triphosphates, 0.4 μM of each primer and 0.5 U of Taq polymerase (Roche Diagnostics). The amplification conditions were as follows: 94°C for 5 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and polymerization at 72°C for 2 min. For each strain, amplified PCR products were digested with the restriction enzymes *Rsa*I and *Cfo*I following the manufacturer's instructions (Fermentas life science, Cinnagen). PCR products were electrophoresed through a 2% agarose gel in 0.5×TBE buffer at 75 V for 2 h and visualized under UV light and compared with reference cultures.

Sequencing and phylogenetic analyses

Genomic DNA was extracted from overnight NA cultures using a DNA extraction kit (Bioneer, Korea). For each bacterial strain, partial *mdh* and *gap*A DNA sequences were amplified with the primers designed to anneal to conserved sequences in the malate dehydrogenase (*mdh*) and glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) genes both of which have previously been shown to be phylogenetically informative at this taxonomic level (Yap et al. 2004; Ma et al. 2007; Kim et al. 2009).

The PCR reactions were performed in a total volume of 50 μl consisting of 20 ng of template DNA, 0.4 U of Taq polymerase (Biotools), 5 μl of 10×PCR buffer, 1.5 mM $MgCl_2$, 200 μM of deoxynucleoside triphosphates, and 0.4 μM of each primer. PCR amplifications were carried out in a S1000™ thermal cycler (Bio-Rad Laboratories, Inc.) with the following steps: initial denaturation at 94°C for 5 min, 35 amplification cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 60 s and a terminal extension phase at 72°C for 7 min. Primers used to amplify the *mdh* and *gap*A gene are listed in Table 2.

Amplified DNA was detected by electrophoresis in 1% agarose gel and stained with ethidium bromide. The PCR products were purified by QIAquick PCR purification kit (Qiagen), and were sequenced using an Automatic Sequencer 3730× (Macrogen Inc., Korea).

Nucleotide sequences for each gene were aligned in Clustal X 1.83 (Thompson et al. 1997) with gap opening penalty of 10 and gap extension penalty of 0.2 for multiple alignments. The initial alignments were refined with BioEdit v7.0.9 (Hall 1999). For phylogenetic comparison, partial *mdh* and *gap*A sequences from enterobacteria related to soft rot *Pectobacteria* were retrieved from the GenBank database. Phylogenetic and Molecular evolutionary analysis were conducted using MEGA 4 (Tamura et al. 2007) by Maximum likelihood and Maximum parsimony methods on the combined data sets. Clade stability was assessed by 1000 parsimony bootstrap replication. Published sequences of *Yersinia pestis* strain YP9100 and YPCO92 were used as outgroup in all reconstructions (Ma et al. 2007; Pitman et al. 2009). Sequence data obtained in this study have been deposited in GenBank under accession numbers from HM114242 to HM114271.

Results

Identification of *Pectobacterium* spp. in potato tubers and ornamental plants

A survey of potato storage facilities and greenhouses in north-east Iran was performed in 2007–2008. In total, 18 isolates from rotted tubers of six different warehouses and 22 isolates from infected ornamental plants of six different greenhouses were all identified as *Pectobacterium* spp. by phenotypic assays and divided in four groups (Table 3).The biochemical tests for production of phosphatase and indole, sensitivity to erythromycin, growth in sodium chloride 5% agreed with those expected for *Pectobacterium* spp. (Gallois et al. 1992; Gardan et al. 2003). The majority of the isolates were determined as *Pectobacterium carotovoum* subsp. *carotovorum* based on biochemical features with few exceptions. Only five isolates from infected potatoes were identified as *P. atrosepticum* (group 1); the rest of the strains isolated from potato were initially described as atypical *P. carotovoum* subsp. *carotovorum* because they did not grow at 37°C (group 2). In addition, the strains of this group could not elicit HR on tobacco leaves (Fig. 1). All of the isolates obtained from ornamental plants were identified as *P. carotovoum* subsp. *carotovorum* (Group 3 and 4). The few isolates that belonged to group 3 differed from reference strains in two tests including producing acid from α-methylglucoside and utilization of D- Glucuronate (Table 3).

Molecular identification

All *Pectobacterium* strains which had been classified by biochemical assays, were further analyzed by PCR to confirm their accordance to those species and subspecies (Table 4). No PCR product typical of *P. carotovorum* subsp. *carotovorum* was produced with EXPCCF/EXPCCR primers in all strains tested except the type strain *P. carotovorum* subsp. *carotovorum* (ATCC 15713). The expected 434 bp PCR-amplified fragment, corresponding to the conserved region of the *P. carotovorum* pectate lyase-encoding gene was obtained in all strains from ornamental plants using Y1 and Y2 primers (group 3 and 4). An identical result was also observed for the type strain (ATCC 15713). Furthermore, all the strains from potato were screened using ECA1f and ECA2r primers. A 690 bp PCR product specific to *P. atrosepticum* was obtained from five of these isolates (group 1). The remaining 13 strains isolated from potato (group 2) have not been amplified with any of species and subspecies–specific PCR primers (Table 4).

All the strains were also subjected to ITS-PCR analysis to confirm the strain identification (Fig. 2 and Table 4). The first group produced the same banding pattern as the type strains *P. atrosepticum* ATCC 33260 and *P. betavasculorum* ATCC 43762. The second group that could not be classified previously by specific PCR, and those isolates that formed the group 3, amplified the same ITS profile as the type strain *P. carotovorum* subsp. *carotovorum* ATCC 15713, *P. carotovorum* subsp. *odoriferum* ICMP 11533 and reference strain *P. wasabiae* SCRI 488. The strains belonging to group 4 produced unique banding patterns different from *Pectobacterium* spp. isolates. The ITS products of all strains were digested with the recommended restriction enzymes for further analysis (Toth et al., 2001) (Table 4). Digestion with *Cof*I restriction enzyme confirmed the identity of the group 1 isolates as *P. atrosepticum* (Fig. 3). Isolates of groups 2 and 3 belonging to potato and ornamental plants respectively, were treated with *Rsa*I and yielded identical RFLP patterns similar to *P. carotovorum* subsp. *carotovorum* type strain ATCC 15713

Table 3 Comparison of phenotypic features of field isolates with those of *Pectobacterium* spp. type and reference strains

Test	Type and reference strains						Potato strains		Ornamental strains	
	Pcc	Pco	Pa	Pb	Pw	Dch	Group1 (n=5)	Group 2 (n=13)	Group 3 (n=7)	Group 4 (n=15)
Pectolytic activity	+	+	+	+	+	+	+	+	+	+
OF	FA[a]	FA	FA	FA	FA	FA	FA	FA	FA	FA
Oxidase	−	−	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+
Indole Production	−	−	−	−	−	+	−	−	−	−
Phosphatase	−	−	−	−	−	+	−	−	−	−
Gelatinase	+	+	−	−	+	+	20[b]	+	+	80
Caseinase	+	+	−	−	+	+	−	+	+	+
Lecithinase	−	−	−	−	−	+	−	−	−	−
Rss[c]	−	+	+	+	−	+	+	−	−	−
Growth at 37°c	+	+	−	+	−	+	−	−	+	+
Sensitivity to Erythromycine	−	−	−	−	−	+	−	−	−	−
Utilizationof: Lactose	+	+	+	+	−	+	+	77	+	+
Trehalose	+	+	+	+	+	−	+	+	+	+
Maltose	−	+	+	+	−	−	+	−	−	−
Palatinose	−	+	+	+	−	−	+	−	−	−
α -Methylglucoside	−	+	+	+	−	−	+	−	29	−
Raffinose	+	+	+	+	−	+	+	84	+	+
Sorbitol	−	+	−	−	−	−	−	−	−	−
α-D(+)-Melibiose	+	+	+	−	−	+	+	+	+	+
D-(+)-Cellobiose	+	+	+	−	+	+	+	+	+	+
D(+)-Arabitol	−	+	−	−	−	−	−	−	−	−
D(-)-Tartrate	−	+	−	−	−	−	−	−	−	−
Citrate (simmons)	+	−	+	−	+	+	+	+	+	+
Malonate	−	−	−	−	−	+	−	−	−	−
D-Glucuronate	+	−	+	+	+	+	+	+	71	+
L-Glutamate	+	+	−	+	−	+	−	+	+	+

[a] Facultatively anaerobic

[b] Percentage of isolates that tested positive

[c] Reducing substance from sucrose

and *P. carotovorum* subsp. *odoriferum* ICMP 11533 (Fig. 4). Members of the group 4 from ornamental plants generated unique *Rsa*I and *Cfo*I restriction profiles distinct from other *Pectbacterium* species tested, suggesting that these strains may be novel (Figs. 3 and 4)

Pathogenicity tests

The virulence of representative strains of potato was quantified using tuber and stem assays. In the stem rotting assay, symptoms in all plants inoculated with strains belonging to group 1 appeared as external darkening, extending up and downwards on the stem. Sometimes, wilting of the leaves and collapse of stems of inoculated plants were observed (Fig. 5). Furthermore, most of the group 2 isolates showed typical symptoms of stem rotting, but variation in symptoms severity were observed between replicate plants, leading to intermediate symptoms developing to both sides of the inoculation point. In the tuber rotting assay, the amount of tissue macerated by

Fig. 1 The results of hypersensetive response (HR) by infiltration of tobacco leaves with bacterial strains: 1 IR-Ph1, 2 IR-Po3, 3 IR-S4, 4 IR-Po12, 5 IR-KA6, 6 *P. wasabiae* (SCRI 488), 7 IR-S12 and 8 distilled water

isolates of group 2 was higher than that macerated by any of the strains of group 1, the type strains of *P. carotovorum* subsp. *carotovorum* ATCC 15713, *P. artosepticum* ATCC 33260, and reference one of *P. wasbiae* SCRI 488 ($P<0.05$) (Fig. 6).

Each representative strain isolated from ornamental plants was inoculated to a related host plant. Isolates obtained from cactus (*Cereus tetragonus*) were inoculated to this plant which showed considerable watery decay (Fig. 7a). Symptoms in other plants inoculated with strains belonging to group 3 and 4 appeared as water soaked lesions with yellow surrounding, developing to necrotic leaf spot (Fig. 7b, c, d). No symptoms developed on mock-inoculated controls.

Phylogenetic analysis of *mdh* and *gap*A sequences

Phylogenetic analysis of representative *Pectobacterium* strains based on *mdh* and *gap*A sequences revealed the presence of four main groups (Fig. 8). Strains of group 1 clustered tightly together with *P. atrosepticum* type strain ATCC 33260 and SCRI 1043 reference one supported with the bootstrap value of 99% (clade II). The members of group 2 from potato tubers grouped with the reference strain of *P. wasabiae* SCRI 488 (clade IV). The presence of *P. wasabiae* strains infecting potato had been reported from New Zealand (Pitman et al. 2008) and United state (Kim et al. 2009) previously.

Isolates belonging to ornamental plants were placed in clade I and III. The first clade formed a separate group with the type strain of *P. carotovorum* subsp. *carotovorum* ATCC15713 and reference strain of *P. carotovorum* subsp. *odoriferum* SCRI 482, while strains in clade III constituted an individual group with the orphan taxon *Ec*106 which had been previously isolated from *Ornithogalum* bulb (Ma et al. 2007).

Discussion

In this paper we identified bacterial strains from potato tubers and ornamental plants using a combination of biochemical and molecular techniques. We examined four methods to differentiate the *Pectobacterium* isolates and found that phylogenetic analysis

Table 4 Description of *Pectobacterium* isolates from Iran based on phenotypic, molecular assays and elicitation of HR on tobacco leaves. NT: not tested

Group	Potato strains		Ornamental strains	
	Group 1 (n=5)	Group 2 (n=13)	Group 3 (n=7)	Group 4 (n=15)
Phenotypic tests	Pa	Pcc	Pcc	Pcc
Specific PCR for Pcc (Y1/Y2)	−	−	+	+
Specific PCR for Pcc (EXPCCR/EXPCCF)	−	−	−	−
Specific PCR for Pa (ECA1f/ ECA2r)	+	−	−	−
ITS-PCR	Pa	Pcc/Pco/Pw	Pcc/Pco/Pw	Atypical
ITS-RFLP (*Rsa*I)	NT	Pcc/Pco	Pcc/Pco	Atypical
ITS-RFLP (*Cfo*I)	Pa	NT	NT	Atypical
HR on tobacco leaves	+	−	+	+

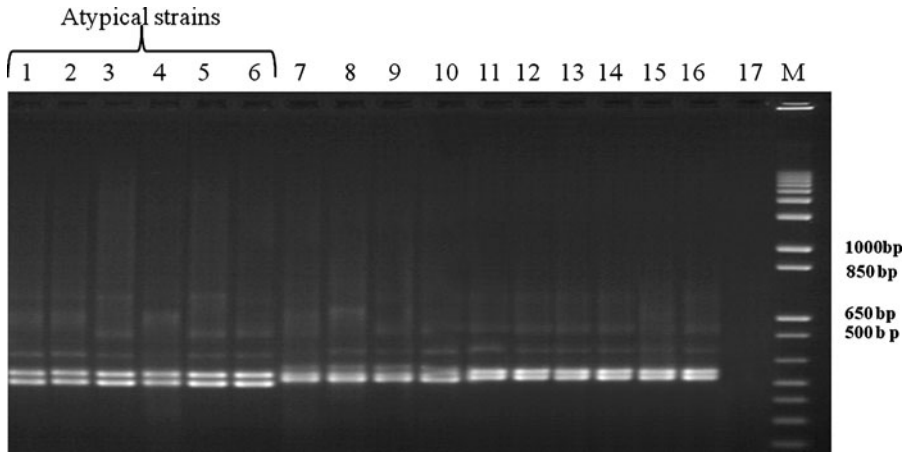

Fig. 2 Characterization of Iranian strains with PCR amplification using universal primers L1G1. Bacterial strains: 1 IR-Is2; 2 IR-S4; 3 IR-S12; 4 IR-KA2; 5 IR-KA8; 6 IR-KA6; 7 IR-Po7, 8 *P. atrosepticum* (ATCC 33260); 9 *P. betavasculorum* (ATCC 43762); 10 *P. wasabiae* (SCRI 488); 11 *P. carotovorum* subsp. *odoriferum* (ICMP 11533); 12 IR-Ph9; 13 IR-AG17; 14 IR-Po3; 15 IR-PoC3; 16 *P. c.* subsp. *carotovorum* (ATCC 15713); 17 distilled water; M 1-kb DNA ladder

of housekeeping genes was the most accurate method to characterize our set of pectolytic enterobacteria.

Most of the isolates were identified as *P. carotovorum* subsp. *carotovorum* using biochemical and physiological experiments. The existence of some unusual *Pcc* strains from potato (group 2) that were unable to grow at 37°C (which also happened in *P. atrosepticum* and *P. wasabi*ae reference strains) and their inability to elicit HR on tobacco leaves persuaded us to check these isolates with other diagnostic tools.

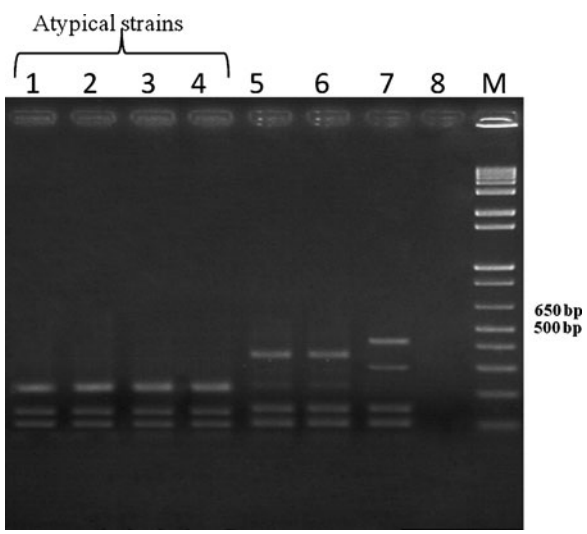

Fig. 3 ITS -RFLP patterns of Iranian *Pectobacterium* strains digested with *Cfo*I enzyme. Bacterial strains: 1 IR-Is2; 2 IR-S4; 3 IR-KA2, 4 IR-KA6; 5 IR-Po7; 6 *P. atrosepticum* (ATCC 33260); 7 *P. betavasculorum* (ATCC 43762); 8 distilled water; M 1-kb DNA ladder

We applied PCR-based technique with specific primers for *P. carotovorum* subsp. *carotovorum* (groups 2, 3 and 4) and *P. atrosepticum* (group 1) to enhance detection sensitivity, simplicity and rapidity in comparison to phenotypic assays. However, thirteen strains were unable to amplify specific bands relevant to *Pectobacterium* spp. (group 2), indicating that some of the field isolates may be novel.

Moreover, we used ITS-PCR analysis following by digestion of PCR products. With these methods, a group of strains from ornamental plants (group 4) previously identified as *P. carotovorum* subsp. *carotovorum* by biochemical and specific PCR techniques, showed a distinct pattern different from the rest of isolates. To further study this group and also those atypical isolates from potato (group 2), we decided to perform phylogenetic analysis of *mdh* and *gap*A genes in a selective subset of strains.

Our phylogenetic results showed four clearly differentiated clades. Clades I, II and IV contained field isolates with type strains that belong respectively to *P. carotovorum* subsp. *carotovorum* ATCC 15713, *P. atrosepticum* ATCC 33260 and *P. wasabi*ae SCRI 488 reference strain, whereas clade III contained field strains from Iran plus strain *Ec*106 described by Ma et al. (2007).

Our results based on phylogeny, fit well with those reported by Ma et al. (2007), indicating that *P.*

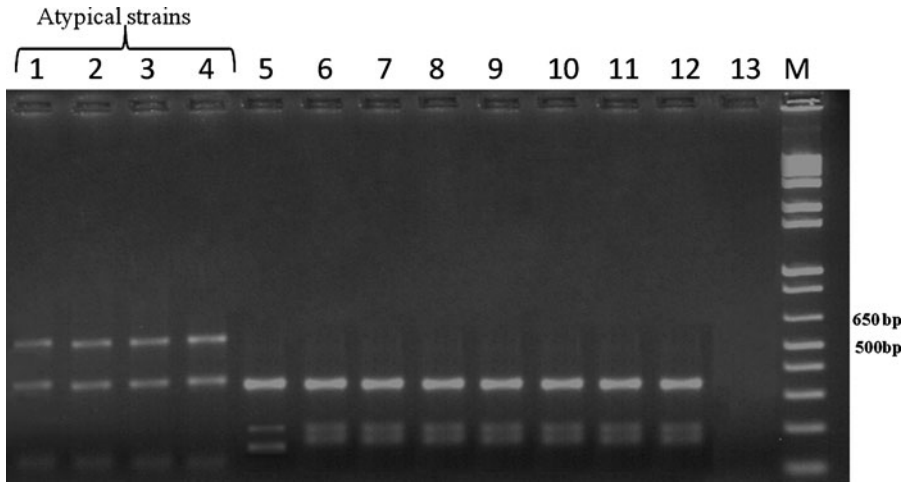

Fig. 4 ITS -RFLP patterns of Iranian *Pectobacterium* strains digested with *Rsa*I enzyme. Bacterial strains: 1 IR-Is2; 2 IR-S4; 3 IR-KA2, 4 IR-KA6; 5 *P. wasabiae* (SCRI 488); 6 *P. carotovorum* subsp. *odoriferum* (ICMP 11533); 7 IR-Ph9; 8 IR-Po3; 9 IR-Po4; 10-IR-PoC3; 11 IR-PoG; 12 *P. c.* subsp. *carotovorum* (ATCC 15713); 13 distilled water; M 1-kb DNA ladder

atrosepticum, *P. betavasculorum*, *P. wasabiae* and *P. brasiliensis* do form individual clades. Phylogenetic outcomes placed *P. carotovorum* subsp. *odoriferum*-SCRI 482 with other *P. carotovorum* subsp. *carotovorum* inside clade I, whereas the level of similarity in this clade does not support maintaining this subspecies here. These results are in agreement with other studies (Ma et al. 2007; Kim et al. 2009).

A group of strains isolated from *Philodendron scandens* and *Aglaonema* spp. were placed in the clade I with *P. carotovorum* subsp. *carotovorum* ATCC-15713. This clade also consisted of strains isolated from potato and marigold reported in previous papers (Ma et al. 2007; Kim et al. 2009) confirming the broad host range and vast geographical distribution of this subspecies. In contrast, clade II contained the *P. atrosepticum* ATCC 33260 type strain with field strains isolated from potato tubers (group 1). This result confirmed the presence of *P. atrosepticum* in Iran by molecular techniques. The genetic differences that limit the host range of *P. atrosepticum* compared with other *Pectobacterium* spp. are unknown (Glasner et al. 2008). It is possible that this species has acquired genes that limit its host range or has lost genes required to cause disease in the diverse range of host plants (Ma et al. 2007). Atypical *P. atrosepticum* has been identified previously in potato (Hélias et al. 1998; Pitman et al. 2008).

Clade III included field isolates from different host plants such as cactus (*Schlumbergera bridgesii)*, iris

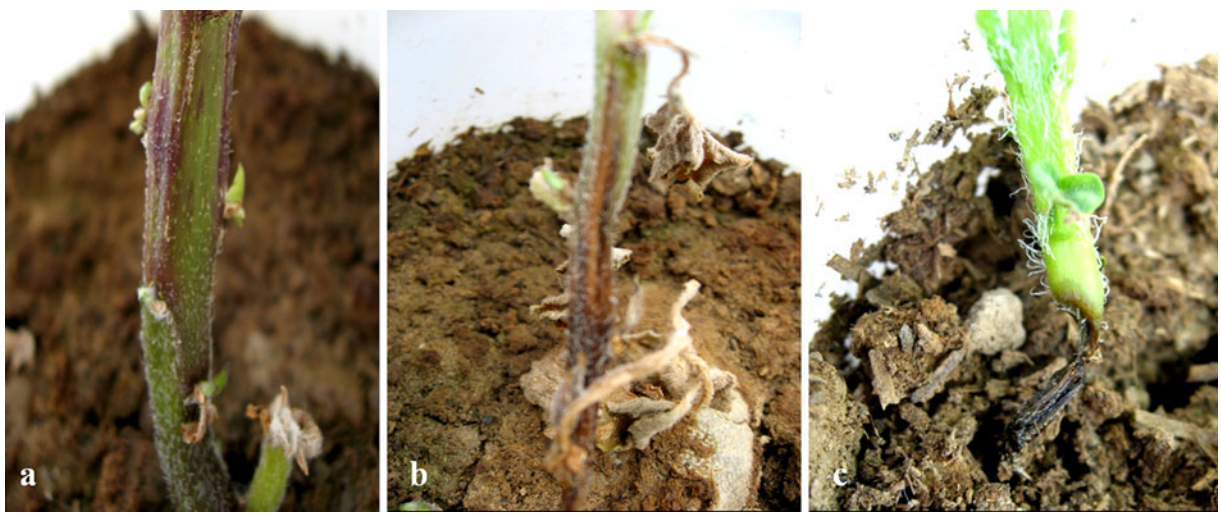

Fig. 5 Symptoms on potato plants cv. Marfana after inoculation of stems with a: Control; b: IR-Po7; c: ATCC33260

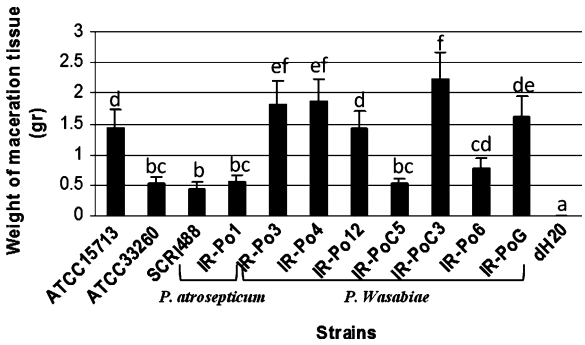

Fig. 6 Relative virulence of *Pectobacterium* strains from Iran and type strains *P. c.* subsp. *carotovorum* (ATCC 15713); *P. atrosepticum* (ATCC 33260) and *P. wasabiae* (SCRI 488) reference strain on potato tubers. The error bars indicate standard errors for five replicates. Bars with the same letter are not significantly different according to a least significant difference test ($P<0.05$)

(*Iris* spp.) and Syngonium (*Syngonium podophyllum)* (group 4). Concurrently, ITS profiles of these strains were identical (Fig. 2). The members of this clade were clearly virulent in greenhouse experiments on ornamental plants and potato tuber slice assay. The phylogenetic analysis confirmed the presence of this clade as a novel group, distinct, but related to the known species of phytopathogenic *Pectobacterium.* Ma and co-workers (2007) proposed a new taxon, from strain *Ec*106, which was isolated from an *Ornithogalum* bulb, placed between *P. atrosepticum* and *P. betavascolorum* clades.

Our phylogenetic analysis showed that the four clades were supported by strong bootstrap values, however, the relative position of clade III can not be ascertained since the bootstrap value for this node was low (Fig. 8).

In agreement with other authors (Yap et al. 2004; Kim et al. 2009; Pitman et al. 2009), the members of clade IV including atypical *Pcc* strains from potato were confirmed as *P. wasabiae* by phylogenetic analysis. This is the first time that the presence of *P. wasabiae* strains from potato in Iran has been described. Strains placed in this clade and *P. wasabaie* SCRI 488 were unable to elicit HR on *N. tabacum* cv *Xanthi.* This conclusion concurred with Kim et al. (2009), while in other investigation, most of the strains belonging to *P. wasabiae* from potato were HR positive (Pitman et al. 2009). No correlation between the intensity of disease symptoms and the incidence of HR was observed. Similar to other researches, *P. wasabiae* isolates of this study were able to cause soft

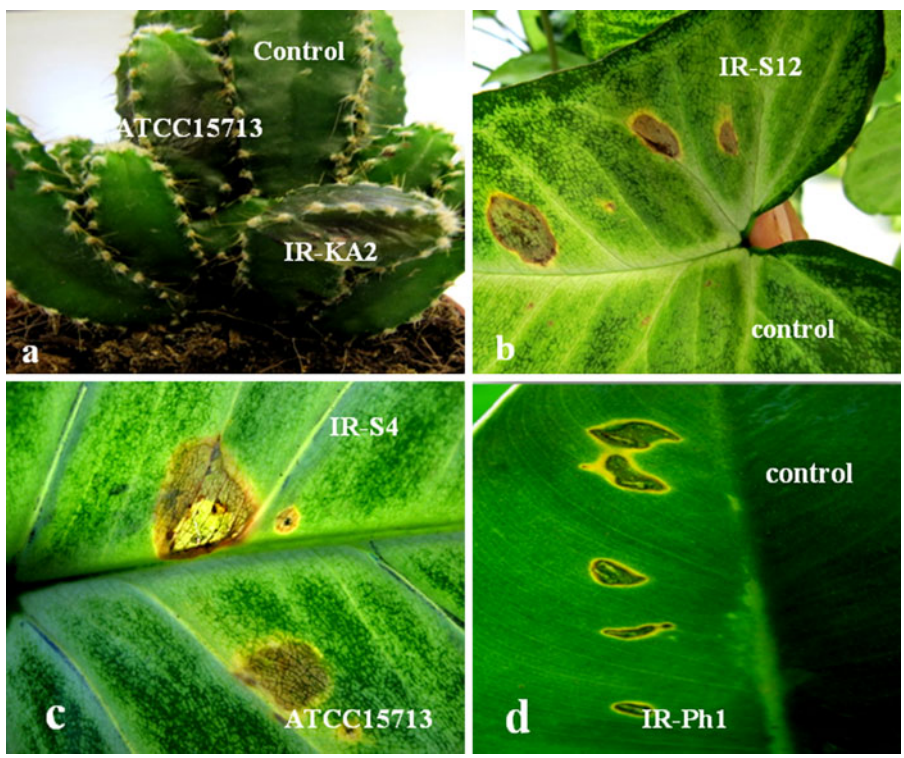

Fig. 7 Symptoms on ornamental plants including a: *Cereus tetragonus;* b and c: *Syngonium podophyllum;* and e: *Philodendron scandens*

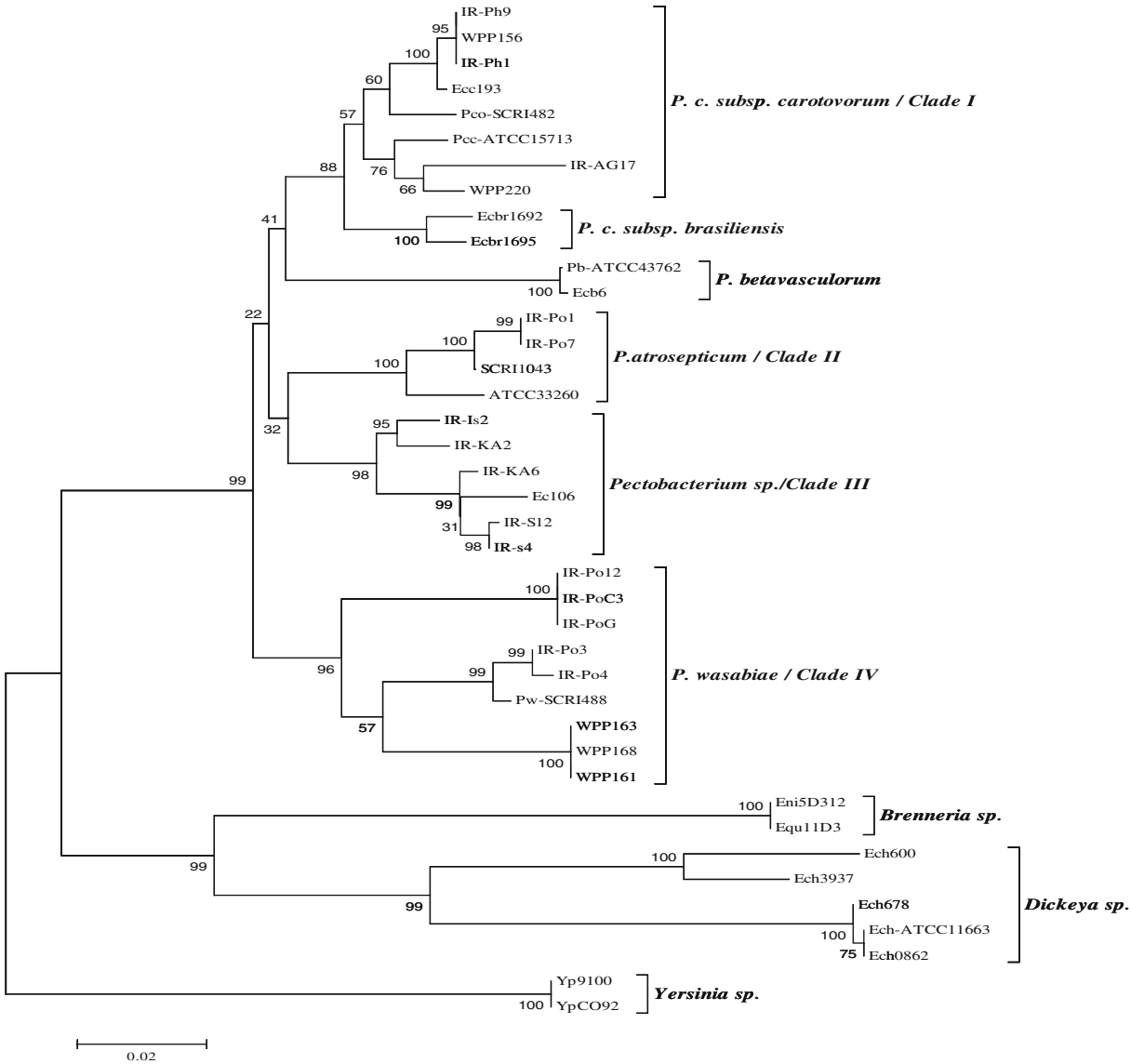

Fig. 8 Maximum likelihood phylogeny analysis of *Pectobacterium* spp. strains from Iran based on *mdh* and *gap*A gene sequences. The strains whose designations begin with ATCC are the type strains of species. *Yersinia* strains were used as outgroups for this analysis

rot of tubers as well as stem lesions (Yap et al. 2004; Pitman et al. 2008). Therefore, it could be hypothesized that different pectolytic species may use different mechanisms to overcome plant barriers.

In the present study, we have shown that phylogenetic analysis with housekeeping genes is a reliable method to classify field strains of *Pectobacterium* spp. This method also was able to differentiate between strains isolated from potato and ornamental plants. In summary, we can conclude that the damaging enterobacteria isolates from potato storages in north-east of Iran belong to *P. wasabiea* and that the group of isolates from ornamental plants (group 4) belongs to a new clade different from other *Pectobacterium* spp. and may therefore constitute a possible new species. However more research will be needed to prove this point. It must be pointed out that this "ornamental" clade containing pectolytic enterobacteria was able to infect both monocot and dicot hosts, so it merits further studies for host specificity, survival and dissemination.

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