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Identification and Genetic Characterization of *Pectobacterium* spp. and Related *Enterobacteriaceae* Causing Potato Soft Rot Diseases in Egypt

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Seventeen soft rot bacteria were isolated from potato tubers and stems showing like symptoms of soft rot and blackleg. Initial characterization concerning morphological and biochemical characteristics was performed. Furthermore, all bacterial isolates showed symptoms of the soft rot disease upon infection of potato tubers with different pathogenic capability. Molecular identification of these isolates via PCR utilizing the 16S rRNA universal primers was carried out. DNA sequence of PCR products and analysis via BLAST and data of the Genbank showed that, twelve isolates belonging to *Pectobacterium carotovorum* sub sp. *carotovorum,* three isolates belonging to *Enterobacter cloacae* and one isolate of each *P. carotovorum* subsp. *brasiliense* and *Dickeya chrysanthemi*. Moreover, the *P. carotovorum* subsp *brasiliense* isolate was confirmed by PCR amplification using specific primers (Br1f/L1r) and represented the expected length 322 bp. The genetic diversity among the seventeen bacterial isolates was investigated by Random Amplified Polymorphic DNA (RAPD) analysis, using 6 different primers, revealed different levels of molecular variation among bacterial isolates on the bases of amplified product pattern. The RAPD analysis shows that isolates of *P. carotovorum* subspecies were quite homogenous and could be differentiated from *Enterobacter. cloacae* isolates.

Key words: *Pectobacterium* spp., Soft Rot *Enterobacteriaceae*, PCR, 16S rRNA, RAPD.

Bacterial soft rot is considered as one of the most frequent diseases observed in different plant species all over the world and causes great total loss of crops (Sherf and Macnab, 1986; Agrios, 1997; Farrar *et al*. 2000). *Pectobacterium* and *Dickeya* species are members of the family *Enterobacteriaceae*. Because of their pectolytic nature, they are generally known as soft rot *Erwinia* or more recently *Enterobacteriaceae* (Charkowski *et al.*, 2012). The soft rot *Enterobacteriaceae* have undergone several run on taxonomic, classification and nomenclature changes and as a result, the former *Erwinia carotovora* and *E. chyrsanthemi* species were subdivided into the genus *Pectobacterium* and *Dickeya* respectively based on biochemical, molecular and host range differences (Hauben *et al*., 1998; Gardan *et al*., 2003; Samson *et al*., 2005).

In general, *Pectobacterium* spp. are broad host range pathogens infecting crop and ornamental plants including both monocots and dicots spanning over 35% angiosperms plant species (Ma *et al*., 2007). *P. carotovorum* subsp. *brasiliensis* was first reported in Brazil in 2004 and later shown to occur in other regions including Israel and USA (Duarte *et al*., 2004; Ma *et al*., 2007). A recent survey of pectolytic bacteria infecting potatoes in Egypt (Behiry, 2013), also identified

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presence of *P. c.* subsp. *brasiliense* and *D. chyrsanthemi.*

Pectobacterium species that have been shown to occur in potatoes are *P. atrosepticum*, *P. wasabiae*, *P. c.* subspp. *carotovorum* and *brasiliense* causing blackleg, tuber soft rot, stem wilt and stem rot symptoms (Kim *et al*., 2009; Pitman *et al*., 2010; van der Merwe *et al*., 2010; Baghaee-Ravari *et al*., 2011). Because there is an overlap in symptoms caused by the soft rot *Enterobacteriaceae*, it is nearly impossible to identify the causal agent by looking at symptoms. Hence biochemical and Polymerase Chain Reaction (PCR) methods of identification are required in order to accurately identify the relevant causal agent.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001)

The phylogenetic relationships between the *Erwinia* species have been investigated by sequence analysis of their 16S rRNA genes (Kwon *et al*., 1997). This study confirmed biochemical observations (Dye, 1981) that *E. carotovora* subspecies and *E. chyrsanthemi* are closely related and form a distinct cluster (cluster III), separate from other *Erwinia* species and from other closely related *Enterobacteriaceae*, such as *E. coli*, which cluster within the *Erwinia* genus. It also provided sequence data with which to develop PCR primers specific to this cluster.

In the last century, diversity within *E. c.* subsp. *carotovora* and *E. chyrsanthemi* strains is well-established and is far greater than that within *E. c.* subsp. *atroseptica*. Therefore, it is possible to relate diversity within *E. chyrsanthemi* and, to a lesser degree, *E. c.* subsp. *carotovora* with host range and geographical location (Maki- Valkama and Karjalinen, 1994; Nassar *et al*. 1996; Parent *et al*. 1996). Different techniques could be used to study genetic diversity present in pathogen population. Random amplified polymorphic DNA (RAPD)-PCR, using low stringency conditions and random primers having short nucleotide sequences has been used efficiently to discriminate genetic diversity among some plant pathogenic bacteria (Mello *et al.* 2006).

The objectives of the present investigation were (i) to isolate and identify soft rot bacteria from potato diseased plants, based on the morphological, biochemical characteristics and PCR of the 16S rRNA gene and (ii) to investigate the genetic variations among *Pectobacterium* spp. and related *Enterobacteriaceae* isolates using RAPD technology.

MATERIALS AND METHODS

Isolation of the soft rot pathogens

Isolation was carried out from naturally infected potato tubers and stems showing soft rot or blackleg symptoms. Potato tubers were collected from different localities of Alexandria and Kafr Al-Sheikh Governorates during 2012-2013 seasons. Diseased potato tubers were washed with tab water several times, surface sterilized by soaking in 1% sodium hypochlorite solution for 2 minutes, rinsed twice in sterilized water, then small portion of the diseased tissues were macerated with 5 mL of sterilized 0.05 M potassium phosphate buffer, after 10 minutes a loopful of the resulting suspension was streaked onto nutrient sucrose agar (NSA) medium (Dowson, 1957). Plates were incubated at 28°C for 48h, and then examined for bacterial growth development. The single colony technique was used to obtain pure culture. Single colony of the isolates was sub-cultured onto the above mentioned medium on tubes and maintained at 4°C for further studies. Also the stock cultures of the isolates were stored in sterilized distilled water at 4°C.

Bacterial isolates and growth media

Bacterial isolates was streaked on nutrient agar (NA) medium in Petri-dishes and incubated at 28°C for 48 h. A single colony of the isolates was selected and grown in 250 mL Erlenmeyer flasks containing 100 mL of nutrient sucrose broth (NSB) and incubated at 28 °C for 48 h. Bacterial cell suspension was centrifuged (8 min. at 10,000 *g*), the cells resuspended in distilled water and cell density adjusted to be $5x10^8$ CFU/mL using

a spectrophotometer at wavelength of 620 nm (McGuire and Kelman, 1984 and Sallam *et al*., 2010). The adjusted bacterial suspensions were used for inoculations.

Pathogenicity and Disease severity

The potato cultivar "Diamont" a commercial variety that are widely grown in Egypt was used for tuber assays. Ability of isolated bacteria to cause soft rot to potato tubers was examined as follows; Uniform size B potato tubers (3.8 to 5.7 cm diameter) were surfaced-sanitized for 10 min with 1% sodium hypochloride solution, rinsed thoroughly, and allowed to air dry. For each isolate, 3 tubers were stabbed between the bud and stem ends approximately 1 cm deep and 0.5 cm wide with a cork-borer, and 100 ìl of a $5x10^8$ CFU/mL bacterial suspension prepared from 24h. culture was placed into the wound then covered with removed potato plugs. Sterile distilled water was used to inoculate negative controls. The potato tubers were placed randomized in plastic trays supplemented with sterilized moist cotton to maintain high humidity, and incubated for 3 days at 28 °C. After incubation, diseased tubers were cut open to observe rotting. (De Boer and Kelman, 1978, Marquez-Villavicencio *et al*., 2011).

Disease severity was estimated as percentage of rotted tissue weight according to change weight of tuber before and after treatment divided on weight of tuber before treatment (Yaganza *et al*., 2004) as following formula: Rot severity = (W1-W2)/W1 ×100.Where, W1= weight of whole tuber and W2= weight of tuber after removal of the rotten tissue. Experiment was set up in a complete randomized design. Data were subjected to analysis of variance (ANOVA), using the statistical analysis system (SAS Institute Inc., 2000). Means were compared with L.S.D. test at $P<0.05$ levels.

Identification of the soft rot bacteria

Phenotypic and Biochemical characterization

The morphological and biochemical characteristics of the isolated bacteria were studied by performing the standard tests recommended by (Dye, 1969; Cowan, 1974; Klement *et al*., 1990 and Staly *et al*., 2005). The above mentioned tests were applied on the obtained seventeen soft rot bacterial isolates and include: Cell shape, Gram staining, motility, anaerobic growth, growth at 36ºC, gelatin liquefaction, indole formation, nitrate reduction, hydrolysis of starch, lipolytic activity, mucoid growth, H_2S production from cysteine, reducing substance from sucrose, acetoin production, urease production, oxidase, growth in 5% NaCl and sensitivity to the antibiotic erythromycin (15µg) as additional test for identification.

Molecular identification

DNA extraction protocol

Bacterial isolates were grown overnight in Luria-Bertani (LB) Agar medium (Miller, 1972) at 28 °C with constant shaking at 200 rpm. Cells from 3 mL culture were pelleted by centrifugation at 6000 *g* for 5 min using a microcentrefuge. Cells of each culture were washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then resuspended in a mixture of 567 µL Tris EDTA, 30 µL of 10% sodium dodecyl sulphate (SDS) and 3 µL Proteinase K (20 mg/mL). After incubation at 37°C for 1 h, 100 µL 5M NaCl and 80 µL of CTAB/NaCl solution were added and the tubes were inverted well before incubation for 10 min in a water bath at 65°C. Phenol/ chloroform/isoamyl alcohol (0.8 mL) was then added, mixed thoroughly and the tubes were centrifuged at 11000 *g* for 5 min. The aqueous supernatant was then taken and the phenol/ chloroform step was repeated one more time. DNA was precipitated by adding equal volume of isopropanol and washed with 70% ethanol. DNA pellets were suspended in 100 µL sterilized distilled water (Ausubel *et al*., 1995).

PCR analyses

One pair of primers (Invitrogen Life Technologies Ltd, Renfrew, UK) was used: BR1f (5'- GCGTGCCGGGTTTATGACCT -3') and L1r (5'- CAAGGCATCCACCGT - 3') based on the DNA probe specific to *Pectobacterium carotovorum* subsp. *brasiliensis* (Duarte *et al*.,2004**).** PCR reactions were carried out in a volume of 25 µl each containing 2µl of template DNA, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 0.5 unit Dynazyme TM II DNA Polymerase (Finnzymes and Finland). Amplifications were performed in the thermal cycler (Techne, UK). The PCR programme consisted of an initial denaturation at 94 ºC for 2 min followed by 25 cycles, each with 45s at 94ºC for denaturation, 45 sec at 62ºC for annealing and 90 sec at 72ºC for elongation. Reaction mixtures were then incubated at 72ºC for 10 min for final

extension. PCR products were separated on a 1.5% agarose gel in TBE buffer (Maniatis *et al*., 1982), stained with ethidium bromide, and photographed under UV light.

Moreover, full length of 16S rRNA gene (1550 bp) was amplified for all bacterial isolates using two universal primers P0 (5'-GAAGAGTTTGATCCTGGCTCAG-3') and P6 (5'-CTACGGCTACCTTGTTACGA -3'). PCR amplification was carried out in a total volume 25 µL containing 2µL of template DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 0.5 unit Dynazyme TM II DNA Polymerase. PCR amplification was performed as one cycle at 95°C for 5 min followed by 34 cycles each with 45 s at 95°C for denaturation, 1 min at 50°C for annealing and 2 min at 72°C for elongation. Reactions were then incubated at 72°C for 10 min for final extension. PCR products were analyzed as above.

Sequencing of 16S rRNA gene

The amplified products of 16S rRNA gene (1550 bp) were purified using Centri-Sep spin columns. The products were sequenced by the use of a Big Dye terminator cycle sequencing kit and resolved on an ABI PRISM model 310 automated DNA sequencer at Sigma Company. Comparisons with sequences in the GenBank database were achieved in BLASTN searches at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov). The 16S rDNA sequences have been deposited in the GenBank database under the accession numbers shown in Table 1.

Random Amplified Polymorphic DNA (RAPD) assay

Six arbitrary primers were used for RAPD analysis (A04, A08, A4, A1A13, A9B7 and USP). Sequences of all primers are shown in Table 2. For RAPD analysis, PCR amplification was carried out in a total volume 25 µL containing 2.5 µL 10x buffer, 2 µL 25 mM $MgCl_2$, 2 µL 25 mM dNTPs, 2 µL 50 ng of bacterial genomic DNA and 0.2 µL (5U.µL^{-1}) Taq DNA polymerase (Promega, Germany). PCR amplification was performed in the thermal cycler (Techne, UK) programmed for one cycle of 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at (35°C for primers A04 and A08; 45°C for primers A4, A1A13 and A9B9), 1 min at 72°C and one cycle of 7 min at 72°C for all tested primers except USP primer. For the USP primer temperature profile was performed by 2 cycles of 94°C for 5 min, 48°C for 5 min and 72°C for 5 min, 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The RAPD-PCR products were separated using 2% agarose gel stained with ethidium bromide then visualized under UV trans-illuminator. Banding patterns of DNA were estimated using TotalLab^M software. Reproducible products were incorporated into cluster analysis using the software PAST 3.02 (Hammer *et al*., 2001).

RESULTS

Isolation and phenotypic characterization

Seventeen bacterial isolates were isolated from infected potato tubers and stems and produced symptoms of soft rot on potato tubers (Table1). Identification of isolated pathogenic bacteria was carried out using the morphological and physiological characteristics presented in Table (3). Out of seventeen isolates, twelve isolates showed to be *P. carotovorum* subsp. *carotovorum* (Pcc), three isolates of *En. cloacae* (Enc) and one isolate of each; *Dickeya. chrysanthemi* (Dch), *P. carotovorum* subsp. *brasiliense* (Pcb) and also based on their pathogenicity tests as presented in Table (1). The above identification was confirmed by PCR analyses.

Pathogenicity assay

Pathogenicity results in Table (1) showed that, the tested seventeen isolates were pathogenic and produced soft rot symptoms on potato tubers. Isolate Pcc14 gave the highest disease index (47.67%) followed by isolate Pcc60 (43.11%) then, the isolate *D. chrysanthemi* (Dch21) (39.80%). From results obtained the disease index of isolate *P. carotovorum* subsp. *brasiliense* (Pcb16) was (37.3%) followed by isolate *En. cloacae* (Enc92) that recorded (36.04%). On the other hand, isolate Pcc10 (22.73%) exhibited weak infection.

Molecular identification through 16S rDNA gene and PCR analyses

The region of the 16S rRNA gene (approximately 1550 bp) was amplified for the 17 bacterial isolates utilizing the universal primers P0 and P6 as stated in materials and methods. The obtained amplicons were purified and sequenced using ABI PRISM model 310 automated DNA sequencer. The BLAST search (http://

www.ncbi.nlm.nih.gov) revealed that, the nucleotide sequences of twelve bacterial isolates were identical to those of *P. c.* subsp. *carotovorum*, three isolates showed to be *En. cloacae* and one isolate of each; *D. chrysanthemi*, *P. c.* subsp. *brasiliense* was identified. All the sequences were submitted to Genbank with accession numbers as illustrated in Table (1).

Genomic DNA isolated from all soft rot isolates were used as template DNA in PCR reaction with the *P. c.* subsp. *brasiliensise* specific primers (Br1f/L1r). The expected amplicon of 322 bp was obtained from only one isolate, Pcb16 (Fig. 1). Whereas, the other tested isolates failed to amplify with this specific primers. Thus, the bacterial isolate (Pcb16) could positively be identified as *P. c.* subsp. *brasiliensise* through species specific primers and confirmed by 16S rRNA gene sequence.

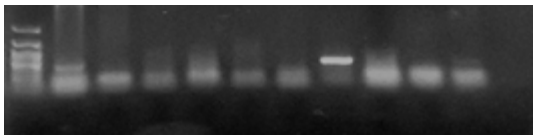

Fig. 1. Electrophoretic pattern of PCR product (322 bp) amplified by specific primers (Br1f/L1r). Lanes 1 to 6 refer to the *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolates, Pcc96, Pcc8, Pcc63, Pcc76, Pcc10 and Pcc7,respectively. Lane7 correspond to the *Pectobacterium carotovorum* subsp. *brasilience* (Pcb) isolate, Pcb16. Lanes 8 to 10 belong to the *Enterobacter cloacae* (Enc) isolates, Enc19, Enc92 and Enc20, in the order given. Lane 11 correspond to the *Dicheya chrysanthemi* (Dch) isolate, Dch21. M, a 1 Kb DNA marker ladder

RAPD assay and cluster analysis

Six primers of arbitrary nucleotide sequences (Table 2) were used to amplify DNA segments for the genomic DNA of twelve isolates of *P. c.* subsp. *carotovorum*, three isolates of *En. cloacae*, one isolate of each *P. c.* subsp. *brasiliense* and *D. chrysanthemi*. The tested primers gave clearly differences among the seventeen bacterial isolates on the bases of amplified product patterns (Fig.2). Results indicated that 255 DNA fragments (bands) were produced by six primers. The comparison between the tested isolates showed differences in the number and molecular length of the amplified fragments produced by each primer for each bacterial isolates. Some bands were common among all tested isolates while others were considered specific to some isolates.

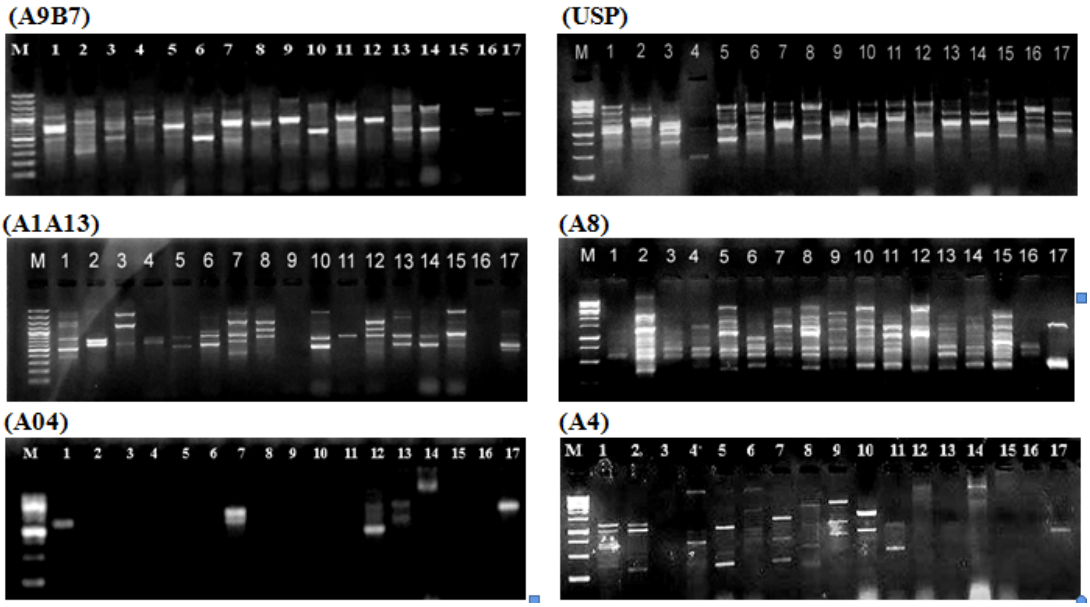

Fig. 2. RAPD-PCR product banding pattern for 17 potato soft rot bacterial isolates with primers: A9B7, A1A13, A04, USP, A8 and A4. Lanes 1 to 12 refer to the *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolates, Pcc96, Pcc14, Pcc9, Pcc8, Pcc63, Pcc76, Pcc60, Pcc6, Pcc79, Pcc93, Pcc10 and Pcc7,respectively. Lane13 correspond to the *Pectobacterium carotovorum* subsp. *brasilience* (Pcb) isolate, Pcb16. Lanes 14 to 16 belong to the *Enterobacter cloacae* (Enc) isolates, Enc19, Enc92 and Enc20, in the order given. Lane 17 correspond to the *Dicheya chrysanthemi* (Dch) isolate, Dch21. M, a 1 Kb DNA marker ladder

The phylogenetic tree generated in this study for the above isolates revealed that two main clusters do exist. Cluster I divided into two Sub-clusters: Sub-cluster I included the isolate Pcb16 of *P. c.* subsp. *brasiliense* interfered with the seven *P. c.* subsp. *carotovorum* isolates, Pcc72, Pcc10, Pcc60, Pcc93, Pcc6, Pcc7, Pcc96. Sub-cluster II included all *En. cloacae* isolates, Enc19, Enc20 and Enc92. Cluster II contained the *D. chrysanthemi* isolate Dch21 and the remaining five isolates of *P. c.* subsp. *carotovorum*, Pcc8, Pcc9, Pcc14, Pcc63 and Pcc79 (Fig.3).

DISCUSSION

Soft rot disease causes huge economic losses estimated to be between 40 to 80% depending on climatic conditions and *P. c.* subsp. *carotovorum* (Pcc) is the causal agent of the soft rot disease of potato tubers in stores and in the field where early decay of mother tubers or seed tuber pieces may occur (Perombelon and Kelman, 1980; Zhijian *et al*., 2000; Chigumira wa Ngwerume, 2002 and Manzira, 2010). In this study; pathogenicity tests of 17 soft rot bacterial isolates were carried out on tubers of "Diamont" potato cultivar. All bacterial isolates showed symptoms of the soft rot disease upon infection of potato tubers with different pathogenic variability. These results were in agreement with those of several authors who pointed out that *P. c.* subsp. *carotovorum* (*E. carotovora*) caused soft rot in potato tubers and other crops (El-Kazazz, 1984; Smith and Bartz, 1990; Saleh and Huang, 1997 and Abd El-Sayed *et al.*, 2003).

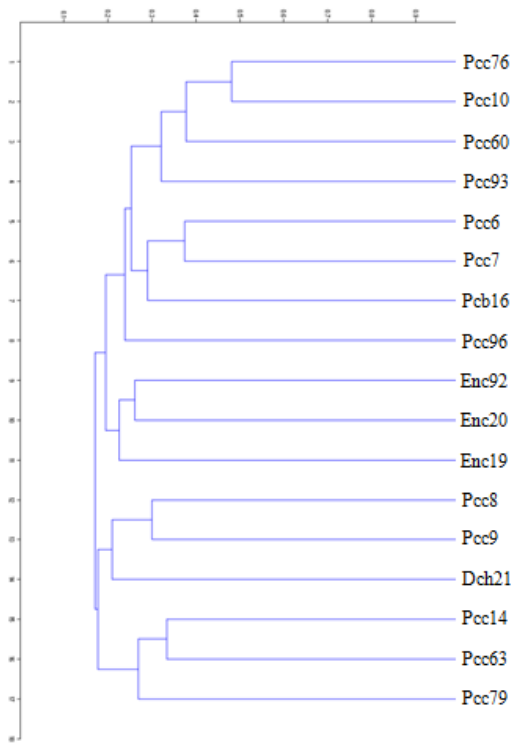

Fig. 3. Dendrogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis of twelve *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolates, three *Enterobacter cloacae* (Enc) isolates and one isolate of each *Pectobacterium carotovorum* subsp. *brasilience* (Pcb) and *Dicheya chrysanthemi* (Dch) according to Jaccard similsrity

The pathological behaviors of the isolated bacterial cultures, as well as, their cultural, morphological and physiological characters were conformity with those known for all soft rot bacteria .On the basis of the obtained data and those reported by Holt *et al*. (1994) and Staley *et al.* (2005) it could identified these isolates as twelve isolates showed to be *P. c.* subsp. *carotovorum*, three isolates of *En. cloacae* and one isolate of each; *D. chrysanthemi*, *P. c.* subsp. *brasiliense* and in agreement with those reported by many workers (Zayed and maayouf, 1989; Choi *et al*., 1990; Duarte *et al.*, 2004 ; Laurila *et al*., 2008 ; Cating and Palmateer, 2009) and recently in Egypt (Behiry, 2013).It could be concluded that, all *P.c carotovorum* isolates were quite homogenous in their biochemical and physiological reactions, the same results were found by Yahiaoui-Zaidi *et al.*(2003) and Baghaee-Ravari *et al.* (2011)

In Egypt *P. atrosepticum* and *P. carotovorum* have been listed as the major pathogens which cause blackleg and tuber soft rot diseases respectively (El-Kazazz, 1984; Abdel-Alim, 1996; Ahmed, 2009 and Behiry, 2009 and 2013). In this study new clades of isolates were identified by biochemical and molecular techniques (16S rRNA sequencing and PCR analysis) as *P. c.* subsp. *brasiliensis*, *En. cloacae* and *D. chrysanthemi* has been isolated from potato tubers with typical soft rot symptoms and this is the second report of a soft rot caused by new isolates on potato in Egypt after first reported by Behiry

(2013). Whereas, it were registered in other countries as a soft rot pathogen in potato, sweet potato, dragon fruit, onion, calla lily *Tolumnia* and *Vanda* Orchids as first report too, or it were concluded in their microbial flora causing that disease (Cating and Hong, 2008; Laurila *et al*., 2008; Awang *et al*., 2009; Cating and Palmateer, 2009; Kowalska *et al.*, 2010 and Zaid *et al*., 2011). Although some of the enterobacterial soft rot pathogens have not been identified in Egypt on potato, their importance as disease agents in the crop should not be underestimated, as typical soft rot symptoms have been observed in experiments involving artificial inoculation of potato tubers. Misdiagnosis of enterobacterial strains in Egypt may also be due to limitations in diagnostic techniques to differentiate bacterial species.

Studies on the genetic variability of phytopathogenic bacteria are important to elucidate possible relationships between certain populations of the pathogen and the area from where they were originally isolated (Scortichini, 2005). The diversity study helps in understanding the structure of pathogen population which is a prerequisite for the control of the disease. If there are differences among the different strains of a plant pathogenic bacterium at genome level, it is of significance in understanding the ecology of the pathogen in a certain area (Seo *et al*. 2002). Moreover, when breeding for resistance to a disease, it is important to have a thorough knowledge of the degree of genetic and pathogenic variation in the pathogen (Arabi and Jawhar, 2007). A huge amount of seed potatoes has been imported from neighboring and overseas countries with reports of increasing

Table 1. Source, accession number and pathogenic capability of bacterial soft rot isolates and related genera obtained from naturally diseased potato tubers.

Bacterial isolates	Code	Accession no.	Potato part	cultivar	Disease index
Pectobacterium cartovorum subsp. *carotovorum*	Pcc96	HF674984	tuber	-	29.74^{de}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc14	LN851556	tuber	-	47.67^{a}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc9	LN851547	tuber	Rosseta	26.28^{ef}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc8	LN851550	tuber	cara	28.05^{e}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc63	HF674987	tuber	Rosseta	23.34^{f}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc76	HF674988	tuber	-	35.89cd
Pectobacterium cartovorum subsp. *carotovorum*	Pcc60	HF674989	tuber	-	43.11^{b}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc6	LN851553	Stem	cara	37.28^{c}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc79	HF674992	tuber	-	30.27^{de}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc93	HF674990	tuber	Spunta	26.59e^{f}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc10	LN851555	tuber	Hermis	22.73^{f}
Pectobacterium cartovorum subsp. *carotovorum*	Pcc7	LN851554	tuber	cara	26.32^{ef}
Pectobacterium cartovorum. subsp.*brasiliense*	Pcb16	LN851552	tuber	cara	37.33^{c}
Enterobacter cloacae	Enc19	LN851548	tuber	Rosetta	29.36^{de}
Enterobacter cloacae	Enc92	HF674994	tuber	Mondial	36.04^{cd}
Enterobacter cloacae	Enc20	LN851551	tuber	Rosetta	30.88^{de}
Dickeya chrysanthemi	Dch21	HF569035	Stem	-	39.80^{a}
	Control				0.00

$LSD_{0.05}$ Disease index =6.14
Data were average of three replicates.
Means with the same letter are not significantly different

Table 2. Sequence and annealing temperature of random primers used in RAPD analysis

Primer	Nucleotide sequence, 5' to 3'	Annealing temperature
A04	ATCAGCGCACCA	35
A08	GCCCCGTTAGCA	35
A4	CGGGAAGTCTTGGATTTTCC	45
A1A13	GGTGACGCAGGGGTAACGCC	45
A9B7	GGTGACGCAGGGGTAACGCC	45
USP	GTAAAACGACGGCCAGT	48/60

Table 3. Morphological traits, physiological and biochemical reactions of *Pectobacterium carotovorum* subsp. *carotovorum* isolates and enteric groups of the family *Enterobacteriacea* obtained from diseased potato tubers

Bacterial isolates	Characteristic																								
	Shape(rods)	Gram staining	Motility	Anaerobic growth	Potato soft rot	Growth at 37°C	Gelatin liquefaction	Mucoid growth	Kovac's oxidase	H_2S from cysteine	Indole production	R. substance from sucrose	Urease production	Growth in 5% NaCl	Sensitivity to erythromycin	Phosphatase	Malonate utilization	Starch hydrolysis	Glucose	µ-methyl glucoside	Maltose	Lactose	L-Arabinose	Dulcitol	Manitol	trehalose
P. c. subsp. *carotovorum* $^{(n=12)}$	+	-	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	+	-	-	a	a	a	a	-
P. c. subsp. *brasiliense*$^{(n=1)}$	+	-	+	+	+	+	+	+	-	+	-	-	-	-	+	-	+	+	a	-	-	a	a	a	a	a
Enterobacter cloacae$^{(n=3)}$	+	-	+	+	+	+	+	+	-	+	-	-	-	+	-	-	+	+	a/g	a	a	a	a	a	a	a
Dickeya chrysanthemi$^{(n=1)}$	+	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	a	-	a	a	a	a	a	-

+ = More than 80% of isolates gave positive reaction - = Less than 20% of isolates gave negative reaction, a=acid, g=gas

incidence and dispersal of important bacterial potato diseases in the main potato growing areas. However, a few studies are available concerning the genetic diversity of these pathogens in Egypt (Behiry, 2009 and 2013). In most cases, the phenotypic characteristics of soft rot bacteria do not match with the traits published in diagnostics key tables. To address this issue, the RAPD-PCR method was selected due to its simplicity. Therefore, it is used to study pathogenic variations (Jawhar *et al*. 2000; Hsiang and Wu, 2000). Although the reproducibility of RAPD fingerprints can be influenced by the reagents, thermocycler (Tyler *et al*. 1997) and intensity of amplicons used to score the fingerprints (Skroch and Nienhuis, 1995), under well-established parameters the results can be very reproducible within a laboratory (Mello *et al.* 2008). Toth *et al*. (1999) evaluated a number of phenotypic and molecular typing techniques for determining diversity in *P.c*. subsp. *atrosepticum* and concluded that among the molecular techniques tested, RAPD analysis is was the most discriminatory.

Our RAPD results showed that, the 17 soft rot isolates gave different banding patterns and diverse for molecular traits contrasting with the biochemical and physiological homogeneity among *P. carotovorum*, similar results were obtained by Yahiaoui-Zaidi *et al*. (2003).

RAPD analysis potentially provides information across the entire genome as it uses non-specific primers which bind randomly to regions over the whole genome. RAPD analysis would even detect smaller changes caused by point mutations, thus offering a higher degree of sensitivity as compared to that obtained by other methods. As compared to other related Pectobacteria (Erwinias) such as *P. c*. subsp. *carotovorum* (*E.c.* subsp. *carotovora*) and *Dicheya (Erwinia) chrysanthemi*, *P. c.* subsp. *atrosepticum* (*E.c*. subsp. *atroseptica*) is relatively homogeneous (Parent *et al.* 1996). Such relatively low levels of genetic diversity may be due to a subspecies having more recent origins, limited geographical distribution and limited host range. Avrova *et al.* (2002) however, found a quite high level of genetic diversity among 59 strains (grouped at 56.6% ± 10.4 % similarity) of *P. c.* subsp. *atrosepticum* using amplified fragment length polymorphism. Other researchers studying genetic differences among isolates of *Enterobacteriaceae* on potatoes also demonstrated a greater diversity of pectolytic Erwinias infecting potatoes than previously thought (Oliveira *et al*. 2003; Yahiaoui-Zaidi *et al*. 2003; Yap *et al.* 2004).

The dendrogram constructed from RAPD analysis of *P. c.* subspecies, *En. cloacae* and *D. chrysanthemi* isolates collected from geographically diverse zones of Egypt revealed that, there are interference between *P.c.* subspecies *carotovorum*, *brasiliense* isolates and *D. chrysanthemi* based on constructed phylogenetic analysis. On the other hand, all *En. cloacae* isolates where clustered in one sub-cluster. Our result was in agreement with previous studies indicated that isolates of *En. cloacae* differed from *Pectobacterium* subspecies and *D. chrysanthemi* based on the *recA* gene sequence (Behiry, 2013). The phylogenetic structure obtained as a result of our study, however, did not correlate with the aggressiveness pattern or the source of the isolate.

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