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Plant Pathology (2019) **68**, 804–815 Doi: 10.1111/ppa.12976

Pathogenicity and molecular phylogenetic analysis reveal a distinct position of the banana fingertip rot pathogen among the *Burkholderia cenocepacia* genomovars

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Banana (*Musa* spp.) is one of the most widely cultivated subtropical fruits around the globe. Banana cultivation has been extensively increased in southeastern Iran over the last two decades. Recently, banana fruits possessing rotten and blackened fingertip symptoms were observed in Sistan-Baluchestan, Iran. Isolation and characterization of the causal agent showed that the pathogen belongs to the multifaceted bacterial species *Burkholderia cenocepacia*. Pathogenicity tests and host range assays showed that the strains were pathogenic on banana, as well as carrot, onion and potato. All the strains were resistant to 50 mg L^{-1} rifampicin and 200 mg L^{-1} copper sulphate. Phylogenetic analysis of 16S rRNA and *recA* gene sequences showed that the strains belong to two different genomovars of *B. cenocepacia* (III-A and III-B), which also include environmental and cystic fibrosis associated strains of the species. The results obtained from *recA* phylogeny were confirmed using multilocus sequence analysis (MLSA), although MLSA showed that the banana strains were clustered as a novel phylogroup among the members of both genomovars. Banana-pathogenic *B. cenocepacia* strains isolated in Iran were different from the strains isolated in Taiwan, as the '*B. cepacia* epidemic strain marker' reported in the Taiwanese strains was absent from Iranian strains. To the authors' knowledge, this is the first MLSA-based study on the banana-pathogenic strains of *B. cenocepacia*. However, further in-depth molecular studies are needed to decipher the relationships between the banana fingertip rot pathogen and the clinical strains of *B. cenocepacia*.

Keywords: Burkholderia cepacia complex, clinical bacteria, cystic fibrosis, multilocus sequence analysis/typing, *Musa* sp.

Introduction

Since the beginning of the current century, commercial production of subtropical fruits has been significantly increased in Iran and over 188 540 tonnes of these fruits (planted in 10 120 ha) were produced in the country in 2015 (Anonymous, 2016). Banana (*Musa* spp.) occupies 69% of all the subtropical fruit cultivation in Iran, with annual production of more than 130 000 tonnes planted in 4100 ha. Almost all (99%) the banana production areas are located in Chabahar, Konarak, Rask and Sarbaz counties in Sistan-Baluchestan province in southeastern Iran (Anonymous, 2016). Banana production is a new industry in the country; hence, the biotic and abiotic constraints affecting yield and quality of this crop remain to be investigated.

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Published online 26 December 2018

In June 2017, banana fruits (cv. Cavendish) displaying symptoms of rotting and blackening that were suspected to be infected with a bacterial pathogen were sent to the Plant Pathology Laboratory in the Baluchestan Agricultural and Natural Resources Research and Education Centre of Sistan-Baluchestan Province in Iran. Subsequently, in the framework of a national quarantine programme (Osdaghi *et al.*, 2015, 2016a) surveys were conducted from September to December 2017 across the banana-growing areas in the region, as well as the local marketplaces where the locally produced banana fruits were sold. Banana fingers with symptoms were observed across several banana-growing farms. The symptoms on banana fingers were apparently similar to those of moko disease caused by *Ralstonia solanacearum* race 2 (Lee *et al.*, 2004; Peeters *et al.*, 2013). In the survey, fruits were found to decay from the fruit stigma toward the edible pulps, but decay never spread into the vascular system within the floral stem. *Ralstonia solanacearum* infects banana cultivars via roots or rhizomes, and the first symptoms are usually the yellowing and wilting of the oldest leaves (Buddenhagen, 1968); however, no wilting or leaf chlorosis was observed in the banana plants bearing the infected fruits.

Plant Pathology

Decay of banana fingertips was reported for the first time in Honduras in 1962 and the causal agent was identified as *Pseudomonas* sp. (Buddenhagen, 1968). Due to the symptom similarities with moko disease, the new disease was called mokillo (Buddenhagen, 1968). When polyphasic taxonomic analysis was performed on members of *Pseudomonas* spp., several, including the banana mokillo pathogen, and *Pseudomonas cepacia*, the causal agent of onion sour skin (Burkholder, 1950) were transferred into a new genus, *Burkholderia* (Yabuuchi *et al.*, 1992). In 2003, Lee *et al.* reported a decaying disease (mokillo) named 'banana fingertip rot' on banana fingers in Taiwan caused by *Burkholderia cepacia*. PCR-RFLP analysis on the sequences of the 16S rRNA region and *recA* gene revealed that the banana fingertip rot pathogen belongs to genomovar III in the *B. cepacia* complex (Bcc; Lee *et al.*, 2003; Lee & Chan, 2007), which was later proposed as a new species, *B. cenocepacia* (Vandamme *et al.*, 2003; Mahenthiralingam *et al.*, 2008). Four molecular markers, comprising the *B. cepacia* epidemic strain marker (BCESM), type III secretion gene cluster (*bcscV*), cable pilin subunit gene (*cblA*) and the insertion sequence IS*1356*, were described as discriminative features between the Bcc strains isolated from onion and banana (Lee & Chan, 2007). However, to date, no in-depth molecular phylogenetic analysis has been performed on the banana fingertip rot pathogen to decipher the phylogenetic position of its strains among the plant pathogenic, as well as environmental/clinical, strains of Bcc.

This study follows a national quarantine programme to generate a distribution map for the previously existing, as well as newly emerging bacterial diseases of vegetables (Sedighian *et al.*, 2014; Osdaghi *et al.*, 2018a,b), annual crops (Osdaghi, 2014; Yaripour *et al.*, 2018), and fruit trees (Zarei *et al.*, 2018) in Iran. The objectives of the present study were to identify and characterize the causal agent of banana fingertip rot disease in southeastern Iran. Pathogenicity and host range assays, phenotypic tests, as well as PCR-based analyses of the pathogenicity-related genes were performed on the bacterial strains. Furthermore, multilocus sequence analysis (MLSA) has been performed to decipher the phylogenetic position of the banana-pathogenic Bcc strains among the environmental and clinical strains of these bacteria.

Materials and methods

Surveys, sampling and bacterial isolation

Banana clusters or individual fingers showing fingertip rot symptoms were collected from commercial fields in Chabahar, Konarak, Rask and Sarbaz counties, as well as the local marketplaces in Sistan-Baluchestan province in southeastern Iran, and brought to the laboratory for further analysis. Banana fingers were surface sterilized by dipping into 0.5% sodium hypochlorite for 20 s followed by two to three rinses in sterile distilled water (SDW). Small pieces of the infected pulp tissues were cut using a sterile scalpel, and macerated in 20 mL SDW in a sterile Petri dish. A loopful of the resulting suspension was streaked onto yeast-extract peptone glucose agar (YPGA) and nutrient agar (NA) media as described by Schaad *et al.* (2001). The plates were incubated at 25–27 °C and examined for the growth of bacterial colonies 48–72 h post-incubation (hpi). Pure cultures of the resulting bacterial strains were obtained by subculturing single colonies and the strains were resuspended in SDW and stored at 4 °C until further use. For long-term storage, the strains were maintained in 15% glycerol at −70 °C.

Phenotypic characterization of bacterial strains

All the purified bacterial strains (Table 1) were subjected to standard biochemical and physiological tests (Schaad *et al.*, 2001). Gram reaction, oxidase and catalase activity, aerobic/anaerobic growth (O/F), colony characteristics on yeast extract-dextrose-calcium carbonate (YDC) agar medium, and growth on 0.1% triphenyl tetrazolium chloride (TTC) were determined. Hypersensitive reaction (HR) was evaluated on tobacco (*Nicotiana tabacum* 'Turkish') and geranium (*Pelargonum graveolens*) leaves using a bacterial suspension from a 48-h-old culture on YPGA medium at a concentration of 10^8 CFU mL^{-1}. Leaves were checked for HR at 24 and 48 hpi. A reference strain of *B. cepacia* (CFBP 1434) was used as a control in all the biochemical and phenotypic tests. Because soft rotting and maceration were the main symptoms on the infected banana fruits, pectinolytic and amylolytic activity of the bacterial strains was evaluated using the methods described by Schaad *et al.* (2001). In brief, a fresh culture of each strain was spotted on starch agar (NA + 0.5% starch) plates, and amylolytic activity was confirmed when a turbid halo was observed around the colony after 48 h of incubation at 27 °C. Pectinolytic activity was confirmed by potato disk tests and the intensity of pectinolytic activity of the strains was assessed by the intensity of rotting on the disks. Furthermore, cellulolytic and proteolytic activities of the strains were assessed using the method described by Yim *et al.* (2012). All the biochemical and phenotypic tests were repeated twice.

Sensitivity to rifampicin and copper sulphate

The use of copper-based chemicals and commercial antibiotics is a primary approach to manage newly emerging bacterial diseases (Kado, 2010; Osdaghi *et al.*, 2016b, 2017). Rifampicin is one of the most commonly used antibiotics in clinical environments in southeastern Iran to combat infections of cystic fibrosis patients suspected to be caused by Bcc (Khanbabaee *et al.*, 2012). Therefore, all the bacterial strains obtained in this study were evaluated for sensitivity to rifampicin and copper sulphate. The tests were performed using the methods as described previously (Osdaghi *et al.*, 2017), and the concentrations used for each of these compounds (Table 1) were chosen based on the normal rate of commercial use of these compounds in agricultural and clinical environments in the region.

Pathogenicity tests and host range

Pathogenicity of the bacterial strains was tested on unripe banana fingers (cvs Cavendish and Harichal) as described previously (Lee & Chan, 2007). In brief, young banana fingers (15–20 cm long) were superficially disinfected with 0.5% sodium hypochlorite and placed in a sterile glass container. Bacterial suspensions of 10^8 CFU mL^{-1} were prepared from 48-h-old cultures grown on YPGA medium. The fresh suspension (100 μL per finger) was injected into the finger's tip, through the centre of the stigma, using a sterile insulin syringe (BD Lo-Dose; Fisher Scientific) and the fingers were kept in the sterile glass containers until

Table 1 Bacterial strains used in this study, their origin, place of isolation, results of pathogenicity tests, detection of pathogenicity determinant genes and sensitivity against rifampicin and copper sulphate.

			Pathogenicity on					Growth on: Copper sulphate (mg L^{-1})				Growth on: Rifampicin (mg L^{-1})			Pathogenicity determinant genes				
Strain	Origin	Place of isolation	Cavendish	Harichal	Onion	Potato	Carrot	20	100	200	400	20	50	200	BCESM	*bcscV*	*cblA*	IS*1356*	Date of isolation
Ba11	Konarak-Zarabad	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	06/2017
Ba12	Konarak-Zarabad	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	06/2017
Ba30	Konarak-Zarabad	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	07/2017
Ba31	Konarak-Zarabad	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	07/2017
Ba32[a] = CFBP 8617	Konarak-Zarabad	Marketplace	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	09/2017
Ba40[a]	Chabahar-Bahoocalat	Marketplace	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	09/2017
Ba44[a] = CFBP 8618	Chabahar-Bahoocalat	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	09/2017
Ba50	Chabahar-Bahoocalat	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	09/2017
Ba80[a]	Rask	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	10/2017
Ba110[a]	Rask	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	11/2017
Ba301	Sarbaz	Field	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	12/2017
Ba714[a]	Chabahar-Kahiri	Marketplace	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	12/2017
Burkholderia cepacia CFBP 1434	USA		+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	

+, positive; –, negative; CFBP, French Collection for Plant-associated Bacteria.
[a]Strains used for multilocus sequence analysis.

10–14 days post-inoculation (dpi). The same number of banana fingers was inoculated with *B. cepacia* (CFBP 1434) using the method described above, while the negative control fingers were inoculated with SDW. In addition, to assess the pectinolytic activity of the bacterial strains isolated in this study, carrot (*Daucus carota* subsp. *sativus*) and potato (*Solanum tuberosum*) slices, as well as onion (*Allium cepa*) bulbs were used to determine the potential host range of the bacterial strains. The onion bulbs were inoculated using the method described above, while the carrot and potato slices were inoculated in sterile Petri dishes as described by Mafakheri *et al.* (2017). For each bacterial strain, five independent repetitions were performed, and the whole experiment was repeated three times.

Molecular phylogenetic analysis

Detection of genes for pathogenicity determinants

PCR tests were performed using four primer pairs (Table 2) to evaluate whether the four molecular markers (BCESM, *bcscV*, *cblA* and IS*1356*), used to discriminate between the Bcc strains isolated from onion and banana, were present in the bacterial strains recovered from banana in Iran. Presence of *bcscV* was determined using the primer pair bcscV-1F/CP11 that was developed for the differentiation of *B. cepacia* genomovars I and III (Parsons *et al.*, 2001). In addition, the primer pairs Bc-cblA-F/Bc-cblA-R, IS1356-A/IS1356-B and BCESM1/BCESM2 were used for the detection of *cblA*, IS*1356* and BCESM, respectively (Lee & Chan, 2007).

Bacterial DNA was extracted using the Expin Combo GP (GeneAll) DNA extraction kit, as recommended by the manufacturer. The quality and quantity of the DNA were evaluated spectrophotometrically using an ND-100 (NanoDrop Technologies) and adjusted to 50 ng μL^{-1}. For PCRs, a Universal PCR kit with *Taq* DNA Polymerase Master Mix Red (Ampliqon A/S) was used according to the manufacturer's recommendations. For each strain, a 25 μL PCR including 50 ng total DNA and 10 pmol of each primer were used. The sequences of the primer pairs and respective annealing temperatures are described in Table 2.

Phylogeny of 16S rRNA and recA *genes*

Phylogenetic analysis of the sequences of the 16S rRNA region and *recA* gene is capable of differentiating the Bcc genomovars, as well as the formally reclassified species of Bcc (Mahenthiralingam *et al.*, 2000; Coenye & Vandamme, 2003). Therefore, phylogenetic analysis was carried out on these sequences from the bacterial strains isolated in this study. The primer pairs UNI2/UNI5 and BCR1/BCR2 (Table 2) were used to amplify the 16S rRNA and *recA*, respectively (Mahenthiralingam *et al.*, 2000). PCR parameters were as described above. The certificated PCR products were sent to Bioneer Corporation (South Korea) to be sequenced via Sanger sequencing technology, and the resulting sequences were analysed with the BLAST program (http://blast.ncbi.nlm.nih.gov/). Sequences of the 16S rRNA and *recA* genes of Bcc strains sourced from diverse environmental/clinical habitats (Mahenthiralingam *et al.*, 2008) were retrieved from the NCBI GenBank and included in the phylogenetic analysis (Baldwin *et al.*, 2005). Also included in the analysis were the *recA* gene sequences of banana fingertip rot strains isolated in Taiwan (B9, CA2, GQ52 and MQ41; Lee & Chan, 2007). Phylogenetic trees were constructed using the maximum-likelihood method with MEGA v. 6.06 software (Tamura *et al.*, 2013). The model of evolution for maximum likelihood analysis was determined using MODELTEST in MEGA v. 6.06 (Hall, 2011). *Burkholderia mallei* was used to root the phylogenetic trees, and they were constructed with bootstrapping (1000 replications).

Multilocus sequence analysis

To obtain a precise overview of the phylogenetic position of the bacterial strains used in this study, six representative strains (indicated in Table 1) were selected for MLSA. The sequences of five housekeeping genes (*atpD*, *gltB*, *gyrB*, *lepA* and *recA*) were used for the MLSA, as recommended for Bcc strains (Baldwin *et al.*, 2005; Spilker *et al.*, 2009). PCR conditions and sequencing procedures were as described above. Sequences of these genes in other Bcc members were retrieved from the Bcc MLST database (http://pubmlst.org/bcc; Jolley & Maiden, 2010) for use in the phylogenetic analysis. The sequences were

Table 2 Primer pairs used in this study.

Primer name	Sequence (5′–3′)	Amplicon (bp)	Annealing temperature (°C)	Target region	References
UNI2	GACTCCTACGGGAGGCAGCAG	1020	60	16S rRNA	Mahenthiralingam *et al.* (2000)
UNI5	CTGATCCGCGATTACTAGCGATTC				
bcscV-1F	GACTGGCAGCGGTTGTTTTCCG	618	57	*bcscV*	Parsons *et al.* (2001)
CP11	GCCCTTCACGAACTTCATC				
Bc-cblA-F	CCAAAGGACTAACCCATGCT	722	55	*cblA*	Sajjan *et al.* (1995)
Bc-cblA-R	ACGCGATGTCCATCACATACAG				
IS1356-A	GGCCCTGAAGAAGGCGATAT	327	60	IS*1356*	Tyler *et al.* (1996)
IS1356-B	TCCGGCGACACCTCGATGCC				
BCESM 1	CCACGGACGTGACTAACA	1400	63	BCESM	Mahenthiralingam *et al.* (1997)
BCESM 2	CGTCCATCCGAACACGAT				
atpD-For	ATGAGTACTRCTGCTTTGGTAGAAGG	756	55	*atpD*	Spilker *et al.* (2009)
atpD-Rev	CGTGAAACGGTAGATGTTGTCG				
gltB-For	CTGCATCATGATGCGCAAGTG	652	60	*gltB*	Spilker *et al.* (2009)
gltB-Rev	CTTGCCGCGGAARTCGTTGG				
gyrB-For	ACCGGTCTGCAYCACCTCGT	738	60	*gyrB*	Spilker *et al.* (2009)
gyrB-Rev	YTCGTTGWARCTGTCGTTCCACTGC				
lepA For	CTSATCATCGAYTCSTGGTTCG	975	55	*lepA*	Spilker *et al.* (2009)
lepA Rev	CGRTATTCCTTGAACTCGTARTCC				
BCR1	TGACCGCCGAGAAGAGCAA	1043	58	*recA*	Mahenthiralingam *et al.* (2000)
BCR2	CTCTTCTTCGTCCATCGCCTC				

concatenated following the alphabetic order of the genes, and phylogenetic trees were constructed for all the individual genes, as well as the concatenated dataset of sequences as described above. The nucleotide diversity, number of mutations, number of haplotypes and minimum number of recombination events were determined using DnaSP v. 5.10 software (Librado & Rozas, 2009). The number of nucleotide differences among the Bcc strains, as well as the strains recovered from banana fruits was also calculated using the same software. A splits-decomposition network was constructed using SplitsTree v. 4.14.4 (Huson & Bryant, 2006). Splits decomposition is a parsimony method that allows a tree-like network structure if conflicting signals are detected in the tree-based phylogeny (Huson & Bryant, 2006).

The sequences were deposited into the GenBank database under the following accession numbers: MH431940–MH431951 for 16S rRNA; MH744771–MH744774, and MH909254–MH909255 for *atpD*; MH744775–MH744778, and MH909256–MH909257 for *gltB*; MH536773–MH536776, and MH909250–MH909251 for *gyrB*; MH536777–MH536780, and MH909252–MH909253 for *lepA;* and MH444496–MH444507 for *recA*. Furthermore, pure cultures of the representative strains recovered from the banana fruits in Iran were deposited in the CIRM-CFBP culture collection (International Centre for Microbial Resources – French Collection for Plant-associated Bacteria) and assigned accession numbers as follows: strain Ba32 as CFBP 8617 and Ba44 as CFBP 8618.

Results

Disease symptoms and incidence

Surveys were conducted across the banana-growing areas, as well as marketplaces in Sistan-Baluchestan Province, southeastern Iran, from June to December 2017. Banana fruits with symptoms were collected in Chabahar (Bahoocalat and Kahiri regions), Konarak (Zarabad region), Rask and Sarbaz counties. Infected fingers were diagnosed externally by their narrow bodies, often being

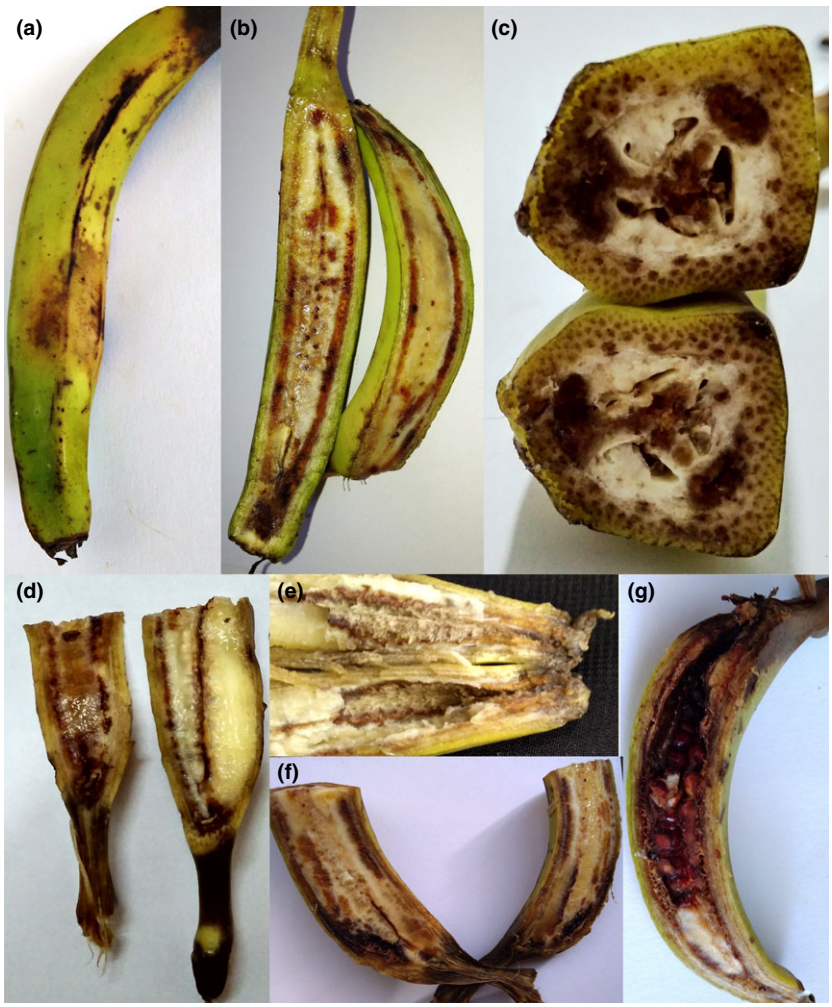

Figure 1 Field symptoms of banana fingertip rot disease caused by *Burkholderia cenocepacia*. Infected fingers have a narrow body and are often smaller and decayed at the tip (a). Fruit pulp is gelatinous and creamy-yellow (b), with a brownish discolouration mostly at the external side of the fruit flesh, just below the skin (c). The brown decay can lead to distortion of the entire finger tissue (d–f) and, in severe infections, the entire flesh of the finger is rotten with a dark brown colour and an unpleasant smell (g). [Colour figure can be viewed at wileyonlinelibrary.com].

smaller and presenting decaying tips (Fig. 1a). Longitudinal cutting of the infected fingers showed that the fruit pulp was gelatinous and creamy-yellow (Fig. 1b). In the cross section of the fingers, brownish discolouration was observed mostly in the external side of the fruit flesh just below the skin (Fig. 1c). Although the brown decay was initially observed at the tip of the flower end, it could lead to distortion of the entire finger tissue during the course of transportation and marketing (Fig. 1d–f). In the case of severe infections, which were mostly seen in the mature clusters in the marketplace, the entire flesh of the finger was rotten with dark brown discolouration and an unpleasant smell, similar to that observed in the odorous soft rot of vegetables caused by *Pectobacterium* species (Fig. 1g). Fortunately, the crop losses from the disease were not severe, and only a few fingers (0.5–2%) in each field showed symptoms.

Phenotypic characteristics of the bacterial strains

White, shiny, mucoid colonies of 1–2 mm in diameter were recovered from the samples collected in the fields and marketplaces. In total, 12 bacterial strains (listed in Table 1) were purified, all of which were Gram-negative and oxidase and catalase positive. All the strains were negative for levan production, but positive for growth on TTC medium, as well as for aesculin, Tween 80, and gelatin hydrolysis. The strains produced acid from D-mannitol, D-sorbitol, fructose, glucose, lactose, maltose and sucrose. All the bacterial strains were positive for pectinolytic and proteolytic activity, while they were negative for cellulolytic and amylolytic activity. Based on the phenotypic characteristics, the bacterial strains recovered from banana fruits were identified as *Burkholderia* sp.

Pathogenicity tests and host range

Banana fingers inoculated with the bacterial strains exhibited typical decay symptoms 10–14 dpi. Initial symptoms on Cavendish were seen 48–72 hpi as a small amount of brownish decaying tissue in the flesh (Fig. 2a,b). However, on Harichal, the infected flesh tissues were pale cream with a sponge-like texture over time (Fig. 2c). The symptoms extended into the finger's centre by 8–10 dpi, when the flesh tissues were entirely decayed and had turned dark brown (Fig. 2d). All the bacterial strains recovered from banana were shown to be pathogenic on potato (Fig. 2e) and carrot (Fig. 2f,g) slices, as well as onion bulbs (Fig. 2h). Interestingly, the intensity of the symptoms (soft rot and decaying) caused by the standard strain of *B. cepacia* (CFBP 1434, isolated from onion) in all the inoculated hosts was more severe than the symptoms caused by banana strains. HR was observed on inoculated tobacco and geranium leaves 24–36 hpi (Fig. 2i).

All the bacterial strains grew on 20, 100 and 200 mg L^{-1} copper sulphate, but were sensitive to 400 mg L^{-1}. In contrast, all the bacterial strains were sensitive to 200 mg L^{-1} but not to 20 and 50 mg L^{-1} rifampicin (Table 1).

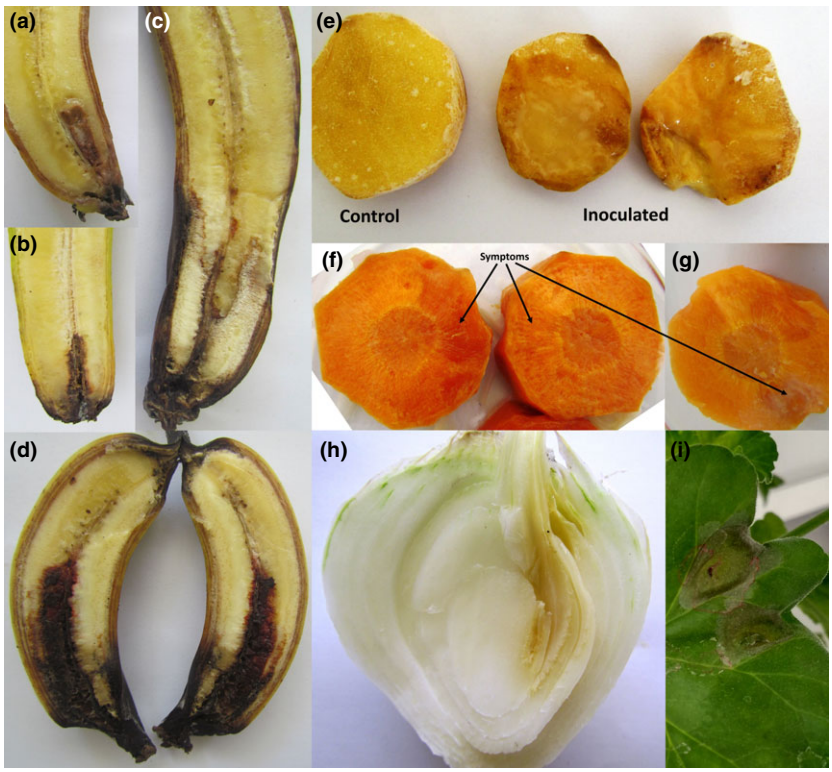

Figure 2 Banana fingertip rot symptoms caused by artificial inoculation with *Burkholderia cenocepacia*. Initial symptoms were seen 48–72 h post-inoculation (a–c). The symptoms extended into the finger's centre 8–10 days post-inoculation (d). All bacterial strains recovered from banana were pathogenic on potato (e) and carrot (f, g) slices, as well as onion bulbs (h). Hypersensitive reaction was observed on geranium (*Pelargonium graveolens*) leaves 24–36 h post-inoculation (i). [Colour figure can be viewed at wileyonlinelibrary.com].

Molecular characterization of the strains

Detection of genes for pathogenicity determinants

PCR results using the bcscV-1F/CP11 primer pair for the detection of the type III secretion gene *bcscV* were positive for all the *Burkholderia* sp. strains recovered from banana fruits, but negative for the onion pathogen *B. cepacia* (CFBP 1434). However, the genes/regions *cblA*, IS*1356* and BCESM were not detected in any of the banana strains or the reference strain CFBP 1434 (Table 1).

Phylogenetic analysis of 16S rRNA and recA *genes*

A BLAST search against the NCBI GenBank database using the 16S rRNA and *recA* gene sequences from the *Burkholderia* sp. strains identified all the strains recovered from banana as *B. cenocepacia* (99–100% sequence similarity). While 16S rRNA phylogeny did not provide a precise distinction among the strains (Fig. S1), the phylogenetic tree that resulted from the analysis of *recA* gene sequences revealed that the *B. cenocepacia* strains isolated from banana belong to two different genomovars of this species (III-A and III-B; Fig. 3). Three strains (Ba80, Ba110 and Ba714) isolated in this study, as well as the fingertip pathogen (B9) isolated in Taiwan were clustered together with the *B. cenocepacia* genomovar III-A. However, nine strains (Ba11, Ba12, Ba30, Ba31, Ba32, Ba40, Ba44, Ba50 and Ba301) isolated in Iran, as well as the fingertip strains from Taiwan (GQ52, CA2 and MQ41) were clustered with *B. cenocepacia* genomovar III-B (Fig. 3).

Multilocus sequence analysis

The phylogenetic tree constructed using the dataset of concatenated sequences of five housekeeping genes confirmed the results obtained from the phylogeny of *recA* gene sequences (Fig. 4). The three strains (Ba80, Ba110 and Ba714) that were identified as *B. cenocepacia*

Figure 3 Phylogeny of *Burkholderia cenocepacia* strains obtained in this study using the *recA* gene sequences. Maximum-likelihood method based on the Tamura 3-parameter model was used. Percentage bootstrap values >50% from 1000 samplings are indicated. *Burkholderia mallei* was used as an out-group cluster. *Burkholderia cenocepacia* strains isolated from banana belong to two different genomovars of the species (III-A and III-B). Three strains (Ba80, Ba110 and Ba714) cluster with the *B. cenocepacia* genomovar III-A, while nine strains (Ba11, Ba12, Ba30, Ba31, Ba32, Ba40, Ba44, Ba50 and Ba301) cluster with *B. cenocepacia* genomovar III-B. [Colour figure can be viewed at wileyonlinelibrary.com].

genomovar III-A based on the *recA* gene phylogeny clustered among the same group in the concatenated tree. Nevertheless, the banana pathogenic strains isolated in Iran were separated from the remaining clinical or environmental strains in the genomovar III-A with a high bootstrap value (100%), and clustered in a novel phylogroup (Fig. 4). Among the non-banana strains in genomovar III-A, the environmental strain POPR8 as well as the cystic fibrosis-associated strain BCC0560 were clustered separately from the other clinical strains in this genomovar. In the same way, although strains Ba32, Ba40 and Ba44 clustered closely to the genomovar III-B of *B. cenocepacia*, they were separated from the clinical and environmental strains of genomovar III-B with a high bootstrap value (99%) and grouped in a unique phylogroup (Fig. 4). Similar results were obtained when the sequences of the individual housekeeping genes (except *atpD*) were subjected to phylogenetic analysis (Fig. S2). When the concatenated dataset of the five housekeeping gene sequences were considered (1872 nucleotides in total), there was a nucleotide difference of 19 nucleotides (1.01%) between Ba80, Ba110 and

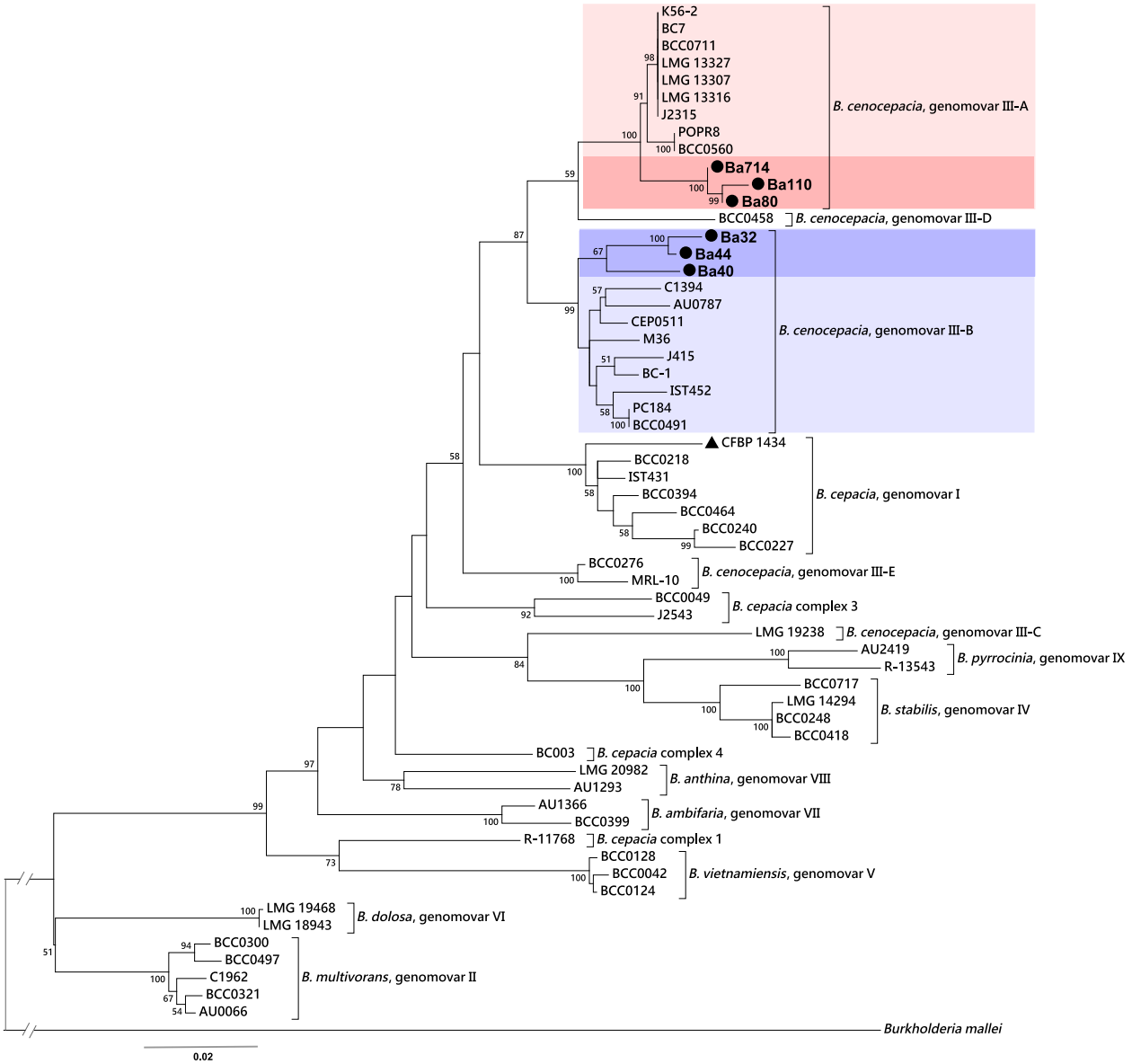

Figure 4 Phylogenetic tree based on the concatenated sequences of *atpD*, *gltB*, *gyrB*, *lepA* and *recA* genes of six representative *Burkholderia cenocepacia* strains obtained in this study. Maximum-likelihood method was used for the construction of the tree using the Tamura–Nei model. Bootstrap scores (1000 replicates) are displayed at each node. *Burkholderia mallei* was used for rooting the tree. Three strains (Ba80, Ba110 and Ba714) clustered as a novel phylogroup in genomovar III-A of *B. cenocepacia*. Similarly, Ba32, Ba40 and Ba44 clustered as a unique group among the genomovar III-B strains of the species. [Colour figure can be viewed at wileyonlinelibrary.com].

Ba714, and the closely related *B. cenocepacia* genomovar III-A strains. Comparison of the *B. cenocepacia* genomovar III-B strains and the strains isolated in Iran (Ba32, Ba40 and Ba44) revealed a difference of seven nucleotides (0.37%). The nucleotide differences among the different genomovars of Bcc are described in Table S1.

No recombination was detected among the banana strains using DnaSP. In all the individual housekeeping genes, the six evaluated banana strains belonged to at least four or more haplotypes. Nevertheless, no nucleotide diversity was observed in the *gltB* and *gyrB* gene sequences of the genomovar III-A strains (Table 3). Because the maximum likelihood phylogenies showed incompatible topologies (Figs 3 & 4), a phylogenetic network was generated using the splits-decomposition method for the dataset of concatenated sequences (Fig. 5). No detectable recombination was observed among the Bcc strains used in this study (Table 3). In the splits-decomposition network, although Ba80, Ba110 and Ba714 were closely clustered with the *B. cenocepacia* genomovar III-A, they can be considered as a unique phylogroup similar to that observed in the maximum likelihood analysis. Strains Ba32, Ba40 and Ba44 were also clustered closely to the *B. cenocepacia*, genomovar III-B group, but could be distinguished within this genomovar (Fig. 5).

Discussion

In this study, field surveys were conducted in the main banana-producing areas of southeastern Iran and a series of phenotypic, molecular and phylogenetic analyses, as well as pathogenicity tests, were performed to characterize the banana fingertip rot pathogen. MLSA-based phylogenetic analysis revealed that two different lineages of *B. cenocepacia* (genomovars III-A and III-B) are responsible for banana fingertip rot disease. To the authors' knowledge, this is the first in-depth phylogenetic analysis on the banana-pathogenic members of *B. cenocepacia*, which provides a novel insight into the relationships between the clinical and plant pathogenic members of this species.

Bcc members have been at the centre of attention for their extraordinary versatility as plant pathogens, saprophytes, biocontrol agents, bioremediation agents, and human pathogens (Parke, 2000). Although they are mostly known as plant pathogenic or opportunistic clinical microorganisms, agriculturally beneficial Bcc strains have also been repeatedly reported in the literature. For example, Bcc members play an important role in the promotion of growth of annual crops (peas, maize, rice and wheat), as well as in the biological control of plant diseases (Parke & Gurian-Sherman, 2001; Mahenthiralingam *et al.*, 2008). These beneficial features resulted in the commercial use of several Bcc strains in the USA (Parke & Gurian-Sherman, 2001). However, no phenotypic/genotypic marker has been identified to differentiate between the beneficial and pathogenic strains of Bcc, nor have distinct phylogenetic differences been observed between these two groups. Accordingly, the opportunistic and clinical nature of Bcc-derived biopesticides led the United States Environmental Protection Agency to place a moratorium on new registrations of products containing Bcc bacteria (https://www.gpo.gov/fdsys/pkg/FR-2004-09-29/html/04-21695.htm; Mahenthiralingam *et al.*, 2008). Although the commercial use of Bcc members has not yet been announced in Iran, the broad host range of *B. cenocepacia* strains recovered from banana reveals that serious caution must be taken prior to development of any Bcc-based biological control in the region.

In addition, isolation of closely related Bcc strains from both human cystic fibrosis patients and plant tissues with symptoms has raised serious concerns (Parke, 2000). For example, a cystic fibrosis-associated strain of *B. cepacia* was identical to the type strain of the species (ATCC 25416) originally isolated from onion rot (Govan *et al.*, 2000). The clinical strain, which was prevalent in the mid-Atlantic region of the USA and Europe, was also abundant in onion field soils in New York State (Lipuma *et al.*, 2002). These findings encouraged the American Phytopathological Society to sponsor a symposium, '*Burkholderia cepacia*: Friend or Foe?' to establish a scientific dialogue among plant pathologists and medical microbiologists (Parke, 2000). As for Iran, so far little is known about the history of *Burkholderia* infections in human and animal hosts (Arefnejad *et al.*, 2013; Soltan Dallal *et al.*, 2014). The results presented here show that the *B. cenocepacia* strains causing banana fingertip rot disease are phylogenetically distinct from the clinical and environmental strains of the species. For sanitary and quarantine reasons, access to the human-pathogenic strains of *B. cenocepacia* was not possible for the present study. Therefore, further in-depth molecular phylogenetic

Table 3 Diversity parameters among the *Burkholderia cenocepacia* strains isolated in this study.

	atpD		*gltB*		*gyrB*		*lepA*		*recA*	
Genomovar	III-A	III-B	III-A	III-B	III-A	III-B	III-A	III-B	III-A	III-B
Number of haplotypes	2	3	1	3	1	2	2	3	2	2
Nucleotide diversity	0.0116	0.0174	0.0000	0.0103	0.0000	0.0193	0.0016	0.0218	0.0073	0.0092
Number of mutations	6	9	0	5	0	13	1	13	4	5
Recombination	0	0	0	0	0	0	0	0	0	0

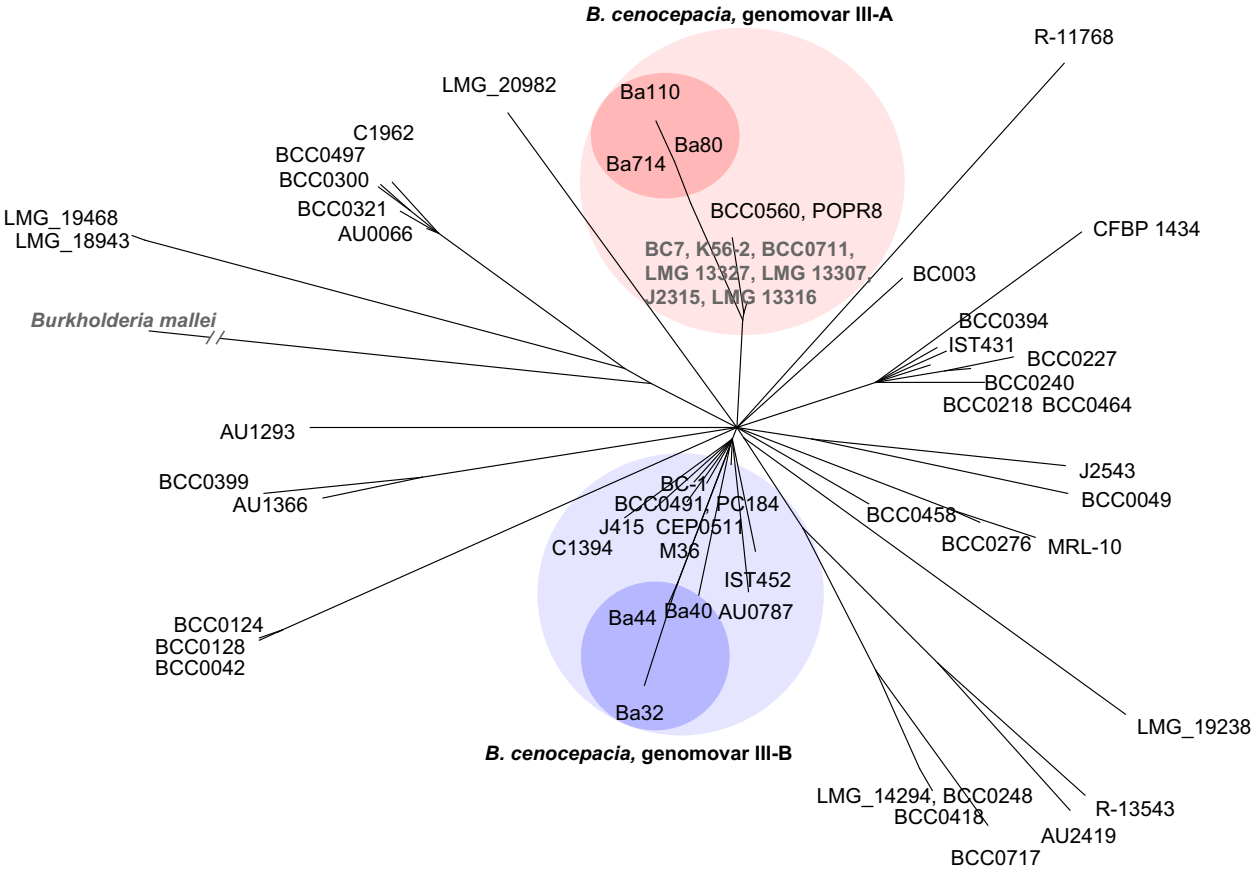

Figure 5 Splits-decomposition network generated from the concatenated sequences of *atpD*, *gltB*, *gyrB*, *lepA* and *recA* genes of *Burkholderia cenocepacia* strains obtained in this study. No detectable recombination was observed among the strains. The strains Ba80, Ba110 and Ba714 were closely clustered with *B. cenocepacia* genomovar III-A, but they can be considered as a unique phylogroup. Likewise, the strains Ba32, Ba40 and Ba44 were clustered closely to the *B. cenocepacia*, genomovar III-B group, but could be distinguished within this genomovar. [Colour figure can be viewed at wileyonlinelibrary.com].

studies are needed to decipher the putative relationships between the plant pathogenic and animal pathogenic strains of the species in southeastern Iran. Mahenthiralingam *et al.* (2008) highlighted the role of the natural environment in the transmission of cystic fibrosis-associated strains of Bcc. Thus, the risk of bacterial transmission from banana fingers to the consumer should be considered. The threat is not restricted to the banana fingers with symptoms as *B. cenocepacia* has been recovered from the stigmata and pulps of banana fingers with no symptoms (Lee *et al.*, 2004). Furthermore, it would be of interest to test the clinical strains of *B. cenocepacia* for banana pathogenicity to determine the interkingdom host range of the pathogen.

It has been noted that almost all Bcc species are highly resistant to commercial antibiotics (Nzula *et al.*, 2002). This was confirmed in the present study where the banana pathogenic strains of *B. cenocepacia* were resistant to 50 mg L^{-1} rifampicin, and 200 mg L^{-1} copper sulphate. Resistance to copper-based antimicrobial compounds is a global challenge for agroecosystems (Lamichhane *et al.*, 2018), although it has not been reported for newly emerging bacterial pathogens in Iran (Osdaghi *et al.*, 2017). Because, thus far, the prevalence of the banana fingertip rot pathogen is restricted to only 0.5–2% of the banana plants in the region, it remains to be clarified whether chemical control is urgently needed to combat this disease. Furthermore, seeking for more sustainable and ecofriendly management methods is warranted, similar to those described for other emerging bacterial diseases in the country (Osdaghi *et al.*, 2010, 2011).

Among the Bcc members, both *B. cenocepacia* (formerly known as genomovar III) and *B. cepacia* (formerly known as genomovar I) can cause soft rot on onion; however, *bcscV*, which is a type III secretion gene, is present in *B. cenocepacia* but absent in *B. cepacia* (Lee & Chan, 2007). However, the PCR patterns for BCESM, cable pilus subunit (*cblA*) and IS*1356* of *B. cenocepacia* strains isolated from banana fruits were different from those of *B. cepacia* strains. Lee & Chan (2007) found that *cblA* and IS*1356* were absent from all the banana strains although BCESM was present; however, this is in contrast with the results of the present investigation, which found that BCESM was absent from all strains isolated in Iran, although present in the banana strains

isolated in Taiwan. A comprehensive complete genome-based study is warranted to shed light on the global diversity of the banana fingertip rot pathogen.

Acknowledgements

This study was financially supported by Shiraz University.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Phylogeny using the 16S rRNA gene sequences of the *Burkholderia cenocepacia* strains isolated in this study and almost all species of *Burkholderia cepacia* complex. Maximum-likelihood method was used and the percentage bootstrap values >50% from 1000 samplings were indicated. *Burkholderia andropogonis* was used as an out-group. The phylogeny confirmed the inclusion of banana strains within the *B. cepacia* complex, although the detailed phylogenetic position of the strains could not be determined.

Figure S2. Phylogenetic trees constructed using the individual sequences of *atpD* (a), *gltB* (b), *gyrB* (c), and *lepA* (d) genes in *Burkholderia cenocepacia* strains obtained in this study. Bootstrap scores (1000 replicates) are displayed at each node. *Burkholderia mallei* was used for rooting the trees. Results obtained from the phylogeny of individual genes were in congruence with those of concatenated sequences.

Table S1. Nucleotide differences among the different species/genomovars of the *Burkholderia cepacia* complex analysed in this study.