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Pectobacterium brasiliense as a Causative Agent for Soft Rot of Radish in Korea

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In October 2021, soft rot disease seriously affected radish crop in Dangjin, Chungcheongnam-do, Korea. The infected radishes were stunted and turned dark green, with yellowish leaf foliage. A slimy, wet, and decayed pith region was observed in the infected roots. The bacterial strain KNUB-03-21 was isolated from infected roots. The biochemical and morphological characteristics of the isolate were similar to those of *Pectobacterium brasiliense*. Phylogenetic analysis based on the sequences of the 16S rRNA region and the concatenated DNA polymerase III subunit tau (*dnaX*), leucine-tRNA ligase (*leuS*), and recombinase subunit A (*recA*) genes confirmed that the isolate is a novel strain of *P. brasiliense*. Artificial inoculation of radish with *P. brasiliense* KNUB-03-21 resulted in soft rot symptoms similar to those observed in infected radish in the field; subsequently, *P. brasiliense* KNUB-03-21 was reisolated and reidentified. To our knowledge, this is the first report of *P. brasiliense* as a causal pathogen of radish soft rot in Korea.

Keywords: Multilocus sequence analysis, Pathogenicity, *Pectobacterium brasiliense*, *Raphanus sativus*, Soft rot

Pectobacterium species, a group of plant pathogenic bacteria, is the causative agent of soft rot disease in crop plants. Monocotyledonous and dicotyledonous plants suffering from bacterial soft rot result in serious economic loss (Ma et al., 2007). Outbreaks of blackleg and soft rot associated with *Pectobacterium brasiliense* have been reported in Korea (Choi and Kim, 2013; Jee et al., 2018; Park et al., 2022). Radish (*Raphanus sativus*) is one of the most widely grown and consumed vegetables worldwide. China is the largest radish producer in the world with planting area of 1.27 million ha and the total production of 44.6 million tons of fresh tuberous roots, accounting for 47% of global radish production (Food and Agriculture Organization, 2019). In Korea, the cultivated area of radish in 2022 was 6,340 ha, which is an increase of 422 ha (7.1%) compared with 5,919 ha in the previous year (Statistics Korea, 2022). Radishes have been cultivated continuously for decades, and they are impacted by diseases such as wilt disease, clubroot, and bacterial soft rot, which deteriorate their quality and affects yield, thereby causing heavy economic damage (Jo et al., 2011; Lee et al., 2018; Moon et al., 2001).

In September–October 2021, the radish crop was severely affected by soft rot disease in Dangjin, Chungcheongnam-do, Korea. During this period, soft rot symptoms (discoloration, decay gray, soft, wet lesions) were observed on radishes in a surveyed 25×64 m field in Dangjin. The disease incidence was found to be around

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15% and the disease severity has attained to moderate level of 10.0% on radishes in the middle and later growth stages. As shown in Fig. 1A and B, the infected roots were discolored and soft rot symptoms were observed on radishes in the middle stage. In addition, soft rot symptoms were observed on the leaves of infected radishes (Fig. 1C, D). The pith region was completely decayed in infected roots in the later growth stage of radishes. Moreover, the inner part of the root contained macerated tissue and emanated a foul smell (Fig. 1E, F). However, the diseased tissue lacked visible fungal mycelia and spores. Small pieces of radish (10×10 mm) were taken, surface-sterilized for 1 min with 1% sodium hypochlorite, and then rinsed with sterile distilled water. A semi-selective crystal violet pectate (CVP) medium, known as the best tool for isolation of pectolytic bacteria such as *Pectobacterium* spp. and *Dickeya* spp. (Boluk et al., 2020; Hélias et al., 2012; Oulghazi et al., 2021), was used to isolate a potential phytopathogenic agent. The collected tissues were macerated in a sterile 1.5 ml microtube with 100 µl sterile water, and approximately 10 µl suspension was spread onto CVP medium. White-gray, round bacterial colonies with entire margins and characteristic cavities in the medium, caused by their ability to metabolize pectin, mainly developed after three days of incubation at 26–28°C. The colonies were repeatedly re-streaked onto nutrient agar plates to obtain a pure culture. As a result, bacterial strain, designated KNUB-03-21, was isolated from an in-

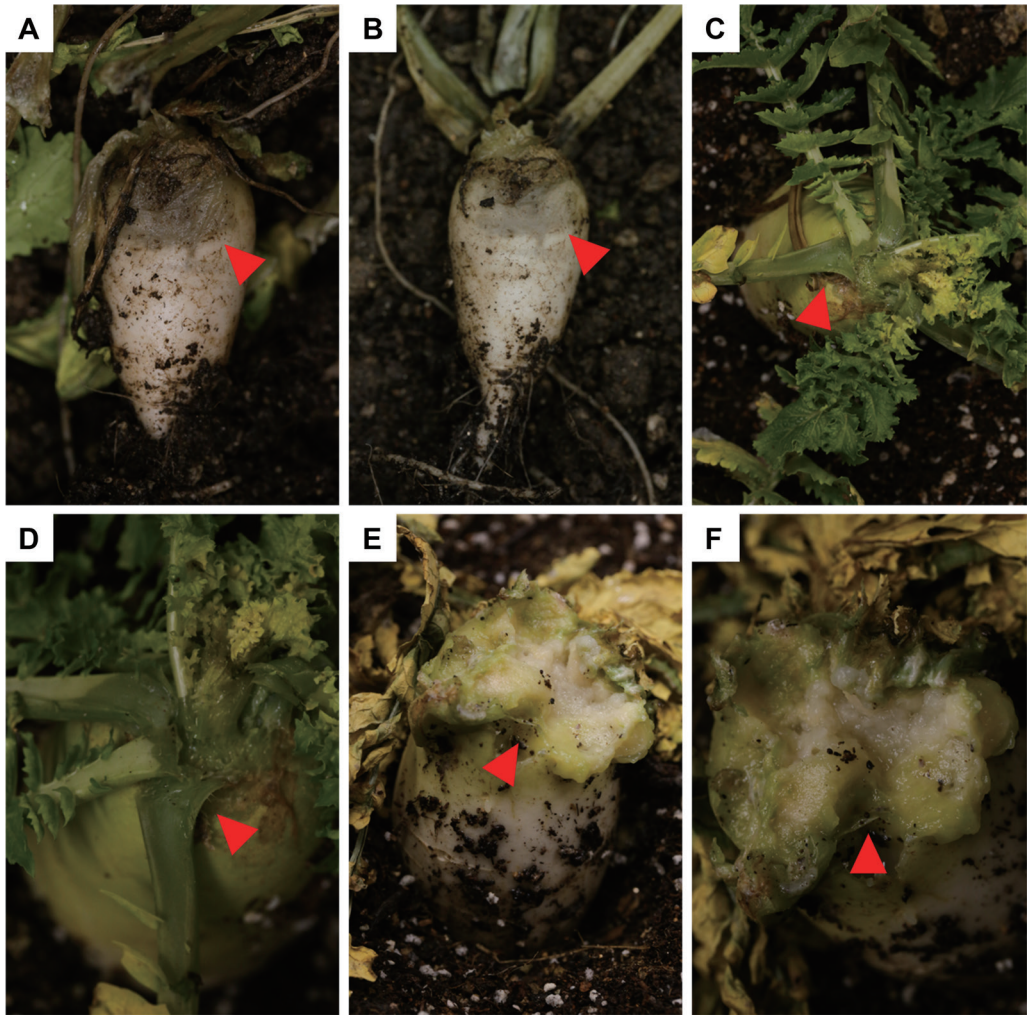

Fig. 1. Radish soft rot symptoms caused by *Pectobacterium brasiliense* KNUB-03-21 in Dangjin, Chungcheongnam-do, Korea. (A, B) The gray, soft, wet lesions were observed on the infected radish root. (C, D) The infected leaves turned dark green and showed soft rot symptoms. (E, F) The inner part of the root contained macerated tissue and emanated a foul smell. Arrowheads indicate the invasion of bacterial cells and soft rot areas.

Table 1. List of PCR primers used in this study

Gene	Primer sequence 5′→3′	Reference
16S rRNA	Forward: GAG TTT GAT CCT GGC TCA G	Weisburg et al. (1991)
	Reverse: ACG GCT ACC TTG TTA CGA CTT	
dnaX	Forward: TAT CAG GTY CTT GCC CGT AAG TGC	Sławiak et al. (2009)
	Reverse: TCG ACA TCC ARC GCY TGA GAT G	
leuS	Forward: TYT CCA TGC TGC CYT AYC CT	Portier et al. (2019)
	Reverse: TCC AGT TRC GCT GCA TGG TT	
recA	Forward: GGT AAA GGG TCT ATC ATG CG	Waleron et al. (2002)
	Reverse: CCT TCA CCA TAC ATA ATT TGG	

PCR, polymerase chain reaction.

Table 2. NCBI BLAST results of the 16S rRNA region sequence and amplicons generated by PCR of three housekeeping genes (*dnaX*, *leuS*, and *recA*) for *Pectobacterium brasiliense* KNUB-03-21

Gene	Amplicon size (bp)	Species	Strain	Similarity (%)	GenBank accession no.
16S rRNA	1,352	*Pectobacterium brasiliense*	PZ7	99.78	MN393965
		Pectobacterium carotovorum subsp. *carotovorum*	PDP201711	99.70	MN394009
		Pectobacterium versatile	ZRIMU1366	99.70	OP476350
dnaX	511	*Pectobacterium brasiliense*	SX309	100	CP020350
		Pectobacterium carotovorum subsp. *carotovorum*	PCC21	100	CP003776
		Pectobacterium atrosepticum	IPO 998	99.02	GQ904832
		Pectobacterium aquaticum	CFBP8637^T	97.11	MK516879
leuS	517	*Pectobacterium brasiliense*	SJDR	99.61	OM321306
		Pectobacterium carotovorum subsp. *carotovorum*	PCC21	99.03	CP003776
		Pectobacterium quasiaquaticum	A398-S21-F17	98.65	CP065178
recA	716	*Pectobacterium brasiliense*	CFBP1350	99.69	MT684213
		Pectobacterium carotovorum subsp. *carotovorum*	PCC21	99.44	CP003776
		Pectobacterium aquaticum	IFB5637	98.04	MW660584
		Pectobacterium quasiaquaticum	A477-S1-J17	98.04	CP065177

Only strains with similarity higher than 97% are shown.

fected radish.

A HiGene Genomic DNA Prep Kit (Biofact, Daejeon, Korea) was used to extract total genomic DNA from strain KNUB-03-21 for molecular analysis. The primers listed in Table 1 were used for polymerase chain reaction (PCR) amplification of the 16S rRNA region and three housekeeping genes: DNA polymerase III subunit tau (*dnaX*), leucine-tRNA ligase (*leuS*), and recombinase subunit A (*recA*). The 16S rRNA gene was amplified as described by Weisburg et al. (1991). A sequence of 1,352 bp was amplified from the 16S rRNA region (GenBank no. LC738895). A BLAST search of the NCBI database revealed 99.70–99.78% similarity between the 16S rRNA region sequence of KNUB-03-21 and several strains belonging to the genus *Pectobacterium* including *P. brasiliense*, *P. carotovorum* subsp. *carotovorum*, and *P. versatile* (Table 2). Based on these results, strain KNUB-03-21 could be recognized as a member of the genus *Pectobacterium*. However, the poor discriminative

resolution of 16S rRNA phylogeny within this genus did not allow accurate identification of the isolate at the species level.

Recently, a phylogenetic analysis using three concatenated housekeeping genes, *dnaX*, *leuS*, and *recA*, allowed to update the taxonomic status of 114 *Pectobacterium* strains and establish *P. actinidiae*, *P. versatile*, *P. odoriferum*, and *P. brasiliense* as the novel species of the genus (Portier et al., 2019). Following this approach, *dnaX*, *leuS*, and *recA* genes of the strain KNUB-03-21 were amplified and sequenced. Briefly, *dnaX* was PCR-amplified as described by Sławiak et al. (2009) and a 511 bp sequence (GenBank no. LC738892) was obtained, which shared 100% identity with phylogenetically closely related species *P. brasiliense* and *P. carotovorum* subsp. *carotovorum*, 99.02% similarity with close phylogenetic relative *P. atrosepticum*, and 97.11% identity with close phylogenetic neighbor *P. aquaticum* (Table 2). The *leuS* gene was amplified as described by Portier et al. (2019) and a sequence of 517 bp (GenBank no. LC738894) was obtained. Based on sequence similarity, the closest relatives of strain KNUB-03-21 were *P. brasiliense* (99.61% similarity), *P. carotovorum* subsp. *carotovorum* (99.03%), and *P. quasiaquaticum* (98.65%) (Table 2). The *recA* gene was amplified as described by Waleron et al. (2002) and a 716 bp fragment (GenBank no. LC738893) was obtained, which showed over 98% similarity with *P. brasiliense* (99.69%), *P. carotovorum* subsp. *carotovorum* (99.44%), *P. aquaticum*, and *P. quasiaquaticum* (98.04%) (Table 2). Similar to the 16S rRNA phylogeny, these results indicated that the comparative analysis based on the sequence of only one of the three loci did not allow precise identification of the closest phylogenetic relatives. Therefore, phylogenetic analysis was conducted using concatenated sequences of three marker genes *dnaX*, *leuS*, and *recA* of strain KNUB-03-21. As mentioned above, these molecular markers have been shown to be highly effective for identifying species within the genus *Pectobacterium* (Portier et al., 2019). For phylogenetic analysis, the related gene sequences of the close phylogenetic relatives were obtained from the NCBI GenBank database (Table 3). The program MEGA7 was used to perform multiple sequence alignment (Kumar et al., 2016). Kimura's two-parameter model and nearest-neighbor interchange heuristic search method were used to perform the maximum-likelihood analysis and construct a phylogenetic tree (Felsenstein, 1981). A monophyletic clade composed of KNUB-03-21 and *P. brasiliense* strains (KNUB-01-21, CFBP5392, CFBP6607, CFBP6615, and CFBP6617^T) with a high bootstrap value strongly suggested that they belong to the same species (Fig. 2). The results of the molecular analysis showed that isolate KNUB-03-21 is a novel strain of *P. brasiliense*.

Biochemical tests were performed to confirm the identity of the isolated strain and the type strain of *P. brasiliense*. Strain KNUB-03-21 could grow at 37°C, formed pits on CVP medium, showed tolerance to 5% NaCl, produced acid from α-methyl glucoside, glucose, and maltose, and formed reducing substances from sucrose. The isolate utilized acetic acid, cellobiose, D,L-lactic acid, D-melibiose, D-sorbitol, succinamic acid, and thymidine as a sole carbon source. However, KNUB-03-21 was negative for assimilation of D-arabitol, 2'-deoxyadenosine, D-glucosaminic acid, D-glucuronic acid, glucose-1-phosphate, inosine, L-glutamic acid, maltose, Tween-40, and Tween-80 and for production of phosphatase. All these characteristics were the same to those of *P. brasiliense* 212^T, but several of them were different in comparison with the type species of the genus *Pectobacterium*, *P. carotovorum* subsp. *carotovorum* 21^T (Meng et al., 2017) (Supplementary Table 1).

Healthy radish root was used to test the pathogenicity of *P. brasiliense* KNUB-03-21. Briefly, the roots were washed under running tap water, air-dried, dipped in 96% ethanol, and cut into 1-cm-thick slices using a scalpel. The slices were placed in Petri dishes and their upper surface was then uniformly covered with *P. brasiliense* KNUB-03-21 inoculum (cell suspension: 10^8 cfu/ml). Rot development on slices was assessed after 48 hr incubation under greenhouse conditions (28°C, 80% relative humidity). The pathogenicity test was repeated twice. In control group, *P. brasiliense* KNUB-03-21 was replaced with sterile distilled water to cover radish slices. Two days after inoculation, soft rot symptoms were observed in radish roots inoculated with KNUB-03-21. In particular, regions of completely decomposed tissue formed on the infected roots (Fig. 3A). In contrast, mock-infected radish slices showed no symptoms (Fig. 3B). The infected radish root was used to re-isolate the pathogen, and *P. brasil-*

Table 3. Pectobacterium species used for phylogenetic analysis and their GenBank accession numbers

Species	Strain no.	GenBank accession no.		
		dnaX	***leuS***	***recA***
Pectobacterium aroidearum	CFBP1457	MT683925	MT684072	MT684219
Pectobacterium aroidearum	CFBP2573	MT683941	MT684088	MT684235
Pectobacterium aroidearum	CFBP6725	MT684029	MT684176	MT684323
Pectobacterium aroidearum	CFBP8737	MT684054	MT684201	MT684348
Pectobacterium atrosepticum	CFBP1526^T	MK516904	MK517048	MK517192
Pectobacterium betavasculorum	CFBP1539^T	MK516905	MK517049	MK517193
Pectobacterium brasiliense	**KNUB-03-21**	**LC738892**	**LC738894**	**LC738893**
Pectobacterium brasiliense	KNUB-01-21	LC717494	LC717495	LC717493
Pectobacterium brasiliense	CFBP5392	MK516927	MK517071	MK517215
Pectobacterium brasiliense	CFBP6607	MK516954	MK517098	MK517242
Pectobacterium brasiliense	CFBP6615	MK516955	MK517099	MK517243
Pectobacterium brasiliense	CFBP6617^T	MK516956	MK517100	MK517244
Pectobacterium cacticida	CFBP3628^T	MK516923	MK517067	MK517211
Pectobacterium carotovorum subsp. *carotovorum*	CFBP1364	MK516896	MK517040	MK517184
Pectobacterium carotovorum subsp. *carotovorum*	CFBP2046^T	MK516909	MK517053	MK517197
Pectobacterium carotovorum subsp. *carotovorum*	CFBP6071	MK516950	MK517094	MK517238
Pectobacterium carotovorum subsp. *carotovorum*	CFBP7351	MK516962	MK517106	MK517250
Pectobacterium odoriferum	CFBP1878^T	MK516907	MK517051	MK517195
Pectobacterium odoriferum	CFBP3259	MK516920	MK517064	MK517208
Pectobacterium odoriferum	CFBP3297	MK516921	MK517065	MK517209
Pectobacterium odoriferum	CFBP5539	MK516929	MK517073	MK517217
Pectobacterium fontis	CFBP8629^T	MK516878	MK517022	MK517166
Pectobacterium parmentieri	CFBP8475^T	MK516972	MK517116	MK517260
Pectobacterium peruviense	CFBP5834	MK516935	MK517079	MK517223
Pectobacterium polaris	CFBP1403	MK516898	MK517042	MK517186
Pectobacterium polaris	CFBP6058	MK516945	MK517089	MK517233
Pectobacterium polaris	CFBP7360	MT684038	MT684185	MT684332
Pectobacterium polaris	CFBP8603^T	MT684046	MT684193	MT684340
Pectobacterium punjabense	CFBP8604^T	MK516877	MK517021	MK517165
Pectobacterium versatile	CFBP1118	MK516888	MK517032	MK517176
Pectobacterium versatile	CFBP2138	MK516912	MK517056	MK517200
Pectobacterium versatile	CFBP6051^T	MK516938	MK517082	MK517226
Pectobacterium versatile	CFBP8656	MK516973	MK517117	MK517261
Pectobacterium wasabiae	CFBP3304^T	MK516922	MK517066	MK517210
Dickeya solani	CFBP7704	MK516970	MK517114	MK517258

The strain isolated in this study is highlighted in bold.
T, the type strain.

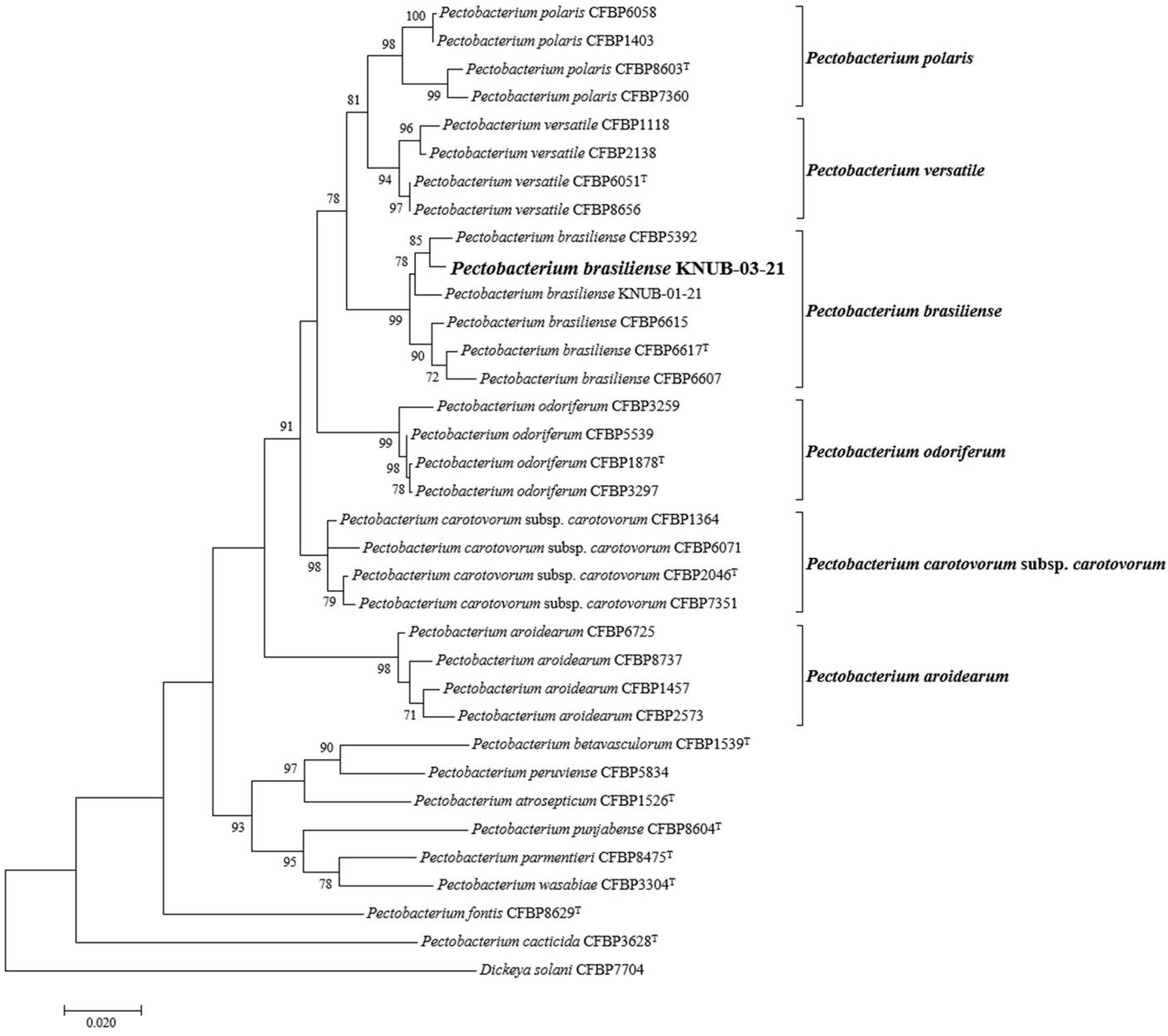

Fig. 2. Maximum-likelihood phylogenetic tree, based on concatenated partial sequences of *dnaX*, *leuS*, and *recA* genes, showing the phylogenetic position of strain KNUB-03-21 among related species of the genus *Pectobacterium*. Bootstrap values (based on 1,000 replications) greater than 70% are shown at branch points. The isolated strain is shown in bold. *Dickeya solani* CFBP7704 was used as the outgroup. Scale bar=0.020 substitutions per nucleotide position.

iense was identified as the isolated bacterial strain (data not shown).

P. brasiliense, one of the main causative agents of soft rot, has been reported to increasingly invade various hosts worldwide. *P. brasiliense* causing soft rot in radish has been reported in China and is known to cause soft rot in 19 different plant species belonging to 10 different families (Liu et al., 2019; Oulghazi et al., 2021).

In this study, we identified and characterized *P. brasiliense* as the bacterial pathogen for soft rot recently observed in radish crop in Korea. To the best of our knowledge, this is the first study reporting *P. brasiliense* as the causal pathogen of radish soft rot in Korea. Our results lay a foundation for the development of control strategies to avert soft rot of plants and related economic losses caused by the identified phytopathogen.

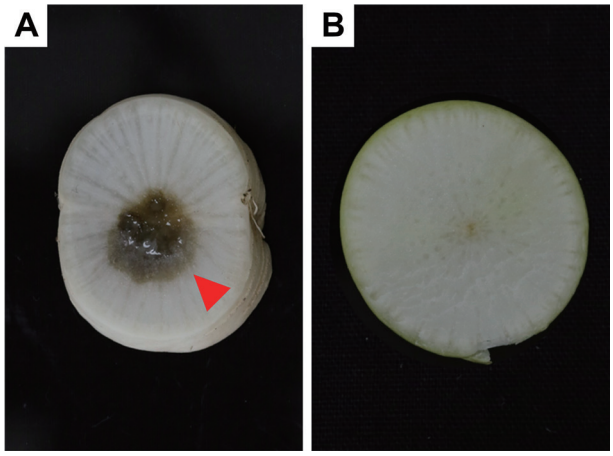

Fig. 3. *Pectobacterium brasiliense* KNUB-03-21 caused radish soft rot. (A) Symptoms of soft rot induced by artificial inoculation with *P. brasiliense* KNUB-03-21. (B) The mock-infected radish slice showed no symptoms. All inoculated radish roots were incubated at 28°C for 48 hr. Arrowhead indicates the invasion of bacterial cells and soft rot areas.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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Electronic Supplementary Material

Supplementary materials are available at Research in Plant Disease website (http://www.online-rpd.org/).

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