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Isolation and identification of the causal agents of blackleg disease of potato occurred in Nagasaki Prefecture, Japan

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Recently, blackleg of potatoes occurred in the fields of Minamishimabara, Shimabara, and Unzen City in Nagasaki Prefecture. Fourteen bacteria were isolated from the potatoes showing blackleg symptoms. When the isolates were inoculated to the stems of potatoes, the original symptoms were reproduced. Moreover, the colonies of reisolated bacteria from the inoculated potatoes showed exactly the same morphological characteristics as the inoculated bacteria. These isolates were analyzed for bacterial and biochemical properties including gram reaction, anaerobic growth, yellow pigment production on yeast dextrose carbonate medium, growth in 5% NaCl, erythromycin sensitivity, acid production from maltose and lactose, utilization of malonate growth in nutrient agar at 37°C, and reduction of sucrose. According to the properties, 12 isolates responded similarly to *Pectobacterium brasiliense* and two isolates did to *P. parmentieri*. The 16S ribosomal RNA, *recA*, and *dnaX* genes were amplified by polymerase chain reaction, and the amplicons were sequenced. Homology search results and phylogenetic analyses based on the nucleotide sequences of the genes supported the identification by the bacterial and biochemical properties. It was concluded that the causal agents of blackleg of potato occurred in Nagasaki Prefecture were identified as *P. brasiliense* and *P. parmentieri*. Since no subsequent outbreaks of the disease have been observed in Nagasaki Prefecture, it is unlikely that the disease was caused by indigenous pathogens. Diversity analysis using various strains worldwide and further epidemiological studies are also required to identify the origin of the pathogens.

Key words: Blackleg disease, Potato, *Pectobacterium brasiliense*, *Pectobacterium parmentieri*

INTRODUCTION

Potato (*Solanum tuberosum*) is one of the world's most important staple foods, providing essential elements such as vitamin C, potassium, and dietary fiber (McGill *et al*., 2013). Potato ranked second in food crop production and was produced in all 47 Prefectures of Japan in 2020. Hokkaido Prefecture contributed around 80% of Japan's over 2.2 million tons of potato production. The next two contributors, Nagasaki and Kagoshima Prefecture, made about 4% each (Japanese Government Statistics: https://www.e-stat.go.jp/). Potatoes are susceptible to various pathogens, that might cause significant losses during cultivation, storage, and processing (Devaux *et al*., 2020). In fact, around 50 pathogens causing potato diseases, including fungi, bacteria, viruses, and nematodes, have been identified in Japan (NARO; https://www.gene.affrc.go.jp/index_en.php).

Bacteria belonging to the *Pectobacterium* and *Dickeya* genera are plant pathogens with a large host range and cause significant economic losses (Ma *et al*., 2007). These pathogens cause several diseases in potatoes at any stage of production. The blackleg potato is caused by six bacterial species including *Pectobacterium atrosepticum, P. brasiliense* (synonym of *P. carotovorum* subsp. *brasiliense*; Portier *et al*., 2019), *P. parmentieri* (synonym of *P. wasabiae* potato isolate; Khayi *et al*., 2016), *Dickeya dianthicola, D. solani*, and *D. chrysanthemi*. The disease is associated with various symptoms, including wilting–chlorosis, stunting, blackening, decay root, and plant death (Pérombelon, 2002).

In Japan, potato blackleg disease was first recorded in 1967 and has been caused by these pathogens, except for *D. solani* (NARO; https://www.gene.affrc.go.jp/index_en.php). Since 1993, the disease has impacted up to 5% of potato production areas annually. In contrast, a recent survey showed that blackleg disease of potatoes occurred in Nagasaki Prefecture in 2018 and 2020. The objective of this study was to identify species of pathogens.

MATERIALS AND METHODS

Isolation of bacterial pathogens

Potato plants showing typical blackleg symptoms were collected from three cities in Nagasaki Prefecture in 2018 and 2020. These samples were washed out of soil and dust with running tap water. A piece of infected tissue was surface sterilized with 3% sodium hypochlorite for 30 sec, then rinsed three times in sterilized distilled water. The tissue was then homogenized in 1 ml sterilized distilled water, and a loopful of suspension was

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streaked on potato semi–synthetic agar (PSA; Wakimoto, 1955). After several rounds of single colony isolation, the bacterial isolates were preserved in 20% (v/v) glycerol solution at –80°C. For routine use, the preserved isolates were streaked on Luria–Bertani (LB) plates, incubated at 30°C for 24 h, and used.

Pathogenicity test

Pathogenicity test was performed by inoculation to about 20–cm high potato plants (cv. "May Queen"). Bacterial cells on the LB plates were picked up with a sterilized toothpick and stab inoculated to the potato stems at 5 cm above the soil line. As a negative control, stems were wounded with a sterilized toothpick in the same manner. The tests were repeated three times. The potato plants were evaluated for blackleg symptoms up to 10 days after inoculation.

Bacterial and biochemical characterizations of the isolates

To identify the isolates, the bacterial and biochemical properties of the isolates were determined, which included gram reaction by 3% KOH solution, anaerobic growth in Hugh and Leifson medium, yellow pigment production on yeast dextrose carbonate (YDC) medium, growth at 37°C, salt tolerance in 5% NaCl, production of reducing sugars from sucrose, and sensitivity to erythromycin (Czajkowski *et al.*, 2015; Schaad *et al.*, 2001). In addition, the utilization of lactose, maltose, and malonate was tested on the basal medium of Ayers *et al.* (1919) supplemented with a corresponding carbohydrates (final concentration, 0.1%).

Homology search and phylogenetic analyses

Isolates were inoculated into 5 ml of LB broth, then cultured under aerobic conditions at 28°C. Genomic DNA was extracted from overnight cultures by the CTAB extraction method described by Wilson (1987).

Three primer pairs, 63f/1387r (Marchesi *et al.*, 1998), dnaXf/dnaXr (Sławiak *et al.*, 2009), and ErecA1/ErecA2 (Waleron *et al.*, 2008), were used to amplify the 16S rRNA, *dnaX*, and *recA* genes, respectively. PCR was performed as reference, but the annealing temperature was modified to 45°C for *recA* gene amplification. After agarose gel electrophoresis, the PCR products were purified using a NucleoSpin Gel and PCR Clean–up kit (Macherey–Nagel, Düren, Germany), and nucleotide sequences were determined at Eurofins Genomics (Tokyo, Japan).

Homology search of the 16S rRNA genes was performed using the BLAST program (https://blast.ncbi.nlm.nih.gov). For phylogenetic analyses, sequences of 16S rRNA and housekeeping genes were aligned using Muscle v5 software (Edgar, 2004), and phylogenetic trees were constructed with the maximum–likelihood phylogenetic method using the IQ–TREE web server (Trifinopoulos *et al.*, 2016). Reference sequences were obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/).

RESULTS

Isolation of the pathogens from potato plants showing blackleg symptoms

The symptoms observed in diseased potato plants were leaf rolling upwards and wilt, darkening and necrotic of the roots and stem base (Fig. 1A, B). Seven isolates from Unzen City, three isolates from Shimabara City, and four isolates from Minamishimabara City were

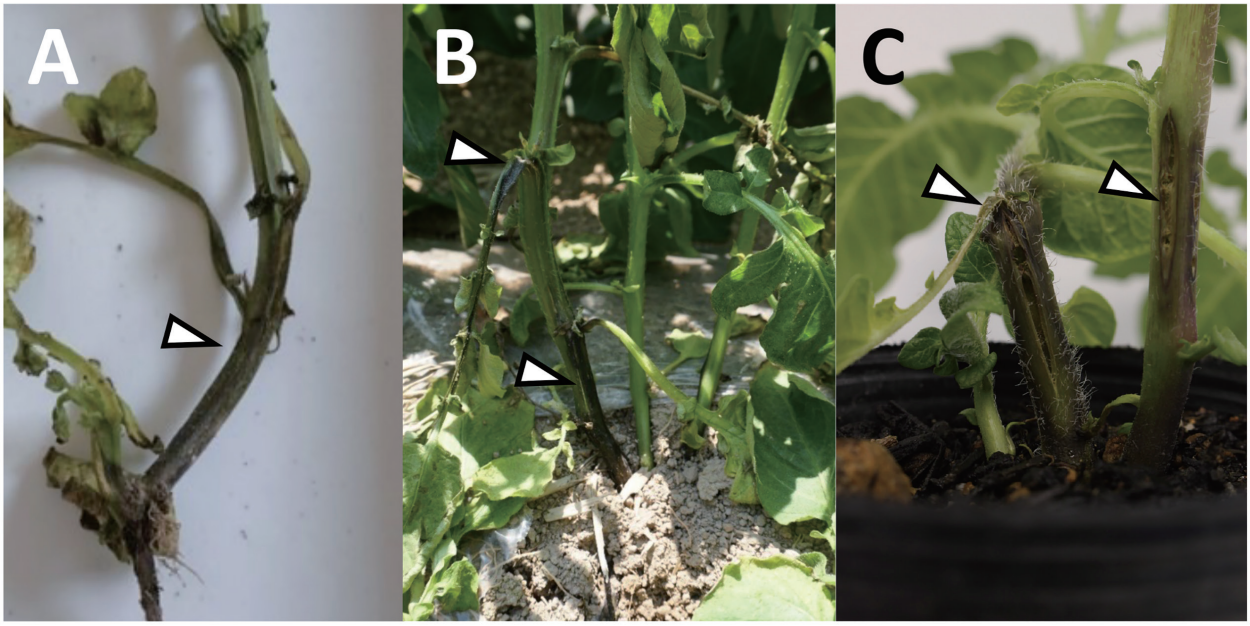

Fig. 1. Symptoms of blackleg disease in naturally infected potatoes in Nagasaki Prefecture (A, B) and in the inoculated potatoes (C). Arrowheads indicate black discoloration of the stems, one of the typical symptoms of the disease.

Table 1. Bacterial isolates from blackleg of potato plants in Nagasaki Prefecture in Japan, and reference strains used in this study

Isolate or strain [1)]	Host	Location (isolated year)	Source [2)]
Present isolates			
KuroAshi 1	*Solanum tuberosum*	Unzen City (2018)	Suga Y.
NK5	*S. tuberosum*	Minamishimabara City (2020)	Suga Y.
NK8	*S. tuberosum*	Minamishimabara City (2020)	Suga Y.
NK9	*S. tuberosum*	Minamishimabara City (2020)	Suga Y.
NK10	*S. tuberosum*	Minamishimabara City (2020)	Suga Y.
NK11	*S. tuberosum*	Shimabara City (2020)	Suga Y.
NK13	*S. tuberosum*	Shimabara City (2020)	Suga Y.
NK14	*S. tuberosum*	Shimabara City (2020)	Suga Y.
NK15	*S. tuberosum*	Unzen City (2020)	Suga Y.
NK16	*S. tuberosum*	Unzen City (2020)	Suga Y.
NK17	*S. tuberosum*	Unzen City (2020)	Suga Y.
NK18	*S. tuberosum*	Unzen City (2020)	Suga Y.
NK19	*S. tuberosum*	Unzen City (2020)	Suga Y.
NK22	*S. tuberosum*	Unzen City (2020)	Suga Y.
Reference strains			
Dickeya chrysanthemi (*Dickeya chrysanthemi* pv. *zeae*)			
MAFF 301657	*Zea mays*	Yamagata Prefecture, Japan	MAFF
MAFF 301658	*Z. mays*	Yamagata Prefecture, Japan	MAFF
MAFF 301659	*Z. mays*	Yamagata Prefecture, Japan	MAFF
Pantoea agglomerans			
LMG 2660	*Wisteria floribunda*	Tokyo Prefecture, Japan	LMG
Pectobacterium carotovorum			
ATCC 15713^T		Denmark	ATCC
Pectobacterium atrosepticum			
ATCC 33260^T	*S. tuberosum*	United Kingdom	ATCC
Pectobacterium betavasculorum			
ATCC 43762^T	*Beta vulgaris*	United States	ATCC
Pectobacterium brasiliense (*Pectobacterium carotovorum* subsp. *brasiliense*)			
MAFF 140116	*S. tuberosum*	Hokkaido Prefecture, Japan	MAFF
Pectobacterium parmentieri (*Pectobacterium wasabiae*)			
MAFF 140134	*S. tuberosum*	Hokkaido Prefecture, Japan	MAFF
Pectobacterium parmentieri (*Erwinia carotovora* subsp. *carorovora*)			
MAFF 211386	*S. tuberosum*	Kagoshima Prefecture, Japan	MAFF
MAFF 301048	*S. tuberosum*	Nagano Prefecture, Japan	MAFF
Pectobacterium wasabiae			
ATCC 43316^T	*Eutrema wasabi*	Shizuoka Prefecture, Japan	ATCC

1) The superscript 'T' indicates the type strain. Scientific names in the database of NARO gene bank are shown in parentheses.

2) Abbreviations of sources are: ATCC, American Type Culture Collection; LMG, Laboratorium voor Microbiologie, Universiteit Gent; MAFF, Ministry of Agriculture, Forestry and Fisheries.

collected and preserved (Table 1). The colony morphologies of these 14 isolates were cream–white, circular, entire, convex, opaque, and smooth on the LB plates.

All 14 isolates caused typical blackleg symptoms 3 to 5 days after inoculation (Fig. 1C). In contrast, any symptoms were not observed in the negative control plants. The colonies of reisolated bacteria from the inoculated potatoes showed exactly the same morphological characteristics as the inoculated bacteria. Namely, Koch's postulates were fulfilled.

Bacterial and biochemical characterizations of the isolates

All 14 isolates were gram–negative, grew anaerobically, did not produce yellow pigments on YDC, were resistant to erythromycin, and grew in 5% NaCl (Table 2). The properties indicated that the isolates belong to the genus *Pectobacterium* (Schaad *et al*., 2001; Czajkowski *et al*., 2015). All isolates were positive in acid production from lactose and negative in malonate utilization, acid production from maltose, and the production of reducing sugars from sucrose. The growth at 37°C test divided the present isolates into two groups; two isolates (NK13 and NK14; group I) were unable to grow at 37°C, but others (group Ⅱ) could. The characteristics of group I and Ⅱ were completely identical to those of *P. parmentieri* and *P. brasiliense*, respectively.

Homology search and phylogenetic analyses

BLAST analysis showed the 16S rRNA genes of the present isolates had high homologies with *Pectobacteirum* species and were included in the *Pectobacterium* clade in the phylogenetic tree (Fig. 2).

In the phylogenetic analysis based on housekeeping genes, isolates in group I constituted a clade with *P. parmentieri*, and group Ⅱ isolates clustered tightly together with *P. brasiliense* in the tree based on the nucleotide sequence of *recA* gene (Fig. 3). Similar results were obtained in the phylogenetic analysis based on *dnaX* (Fig. 3). These results indicated that group I and Ⅱ belong to *P. parmentieri* and *P. brasiliense*, respectively, and verified the identification concluded from the bacterial and biological properties (Table 2).

Accession numbers

Nucleotide sequences determined in this study have been deposited at DDBJ/EMBL/GenBank. The accession numbers for fourteen sequences of each gene, 16S rRNA gene, *dnaX* gene, and *recA* gene, are LC731455 to LC731468, LC731469 to LC731482, and LC731483 to LC731496, respectively.

Table 2. Comparison of bacterial and biochemical characteristics among the present isolates and reference strains

Characteristics	Reaction[1)]									
	Present isolates[2)]		Reference strains							
			Pectobacterium spp.							
	Group I (*n*=2)	Group Ⅱ (*n*=12)	*P. carotovorum*	*P. atrosepticum*	*P. wasabiae*	*P. betavasculorum*	*P. brasiliense*	*P. parmentieri*	*Dickeya*	*Pantoea*
For genus identification										
Gram reaction (3% KOH)	–	–	–	–	–	–	–	–	–	–
Anaerobic growth	+	+	+	+	+	+	+	+	+	+
Yellow pigment on YDC	–	–	–	–	–	–	–	–	–	+
Sensitivity to erythromycin	–	–	–	–	–	–	–	–	+	nt
Growth in 5% NaCl	+	+	+	+	+	+	+	+	–	nt
For species identification										
Utilization of malonate	–	–	–	–	–	–	–	–		
Acid production from lactose	+	+	+	+	–	+	+	+		
Acid production from maltose	–	–	w+	+	–	+	–	–		
Reducing sugars from sucrose	–	–	–	+	–	+	–	–		
Growth at 37°C	–	+	+	–	–	+	+	–		

1) Abbreviations of reactions are: +, positive; w+, weakly positive; –, negative; nt, not tested. For genera *Dickeya* and *Pantoea*, experimental results and descriptions in the literature (Schaad *et al*., 2001; Czajkowski *et al*., 2015) are combined.
2) Group I contains NK13 and NK14, and group Ⅱ consists of other isolates.

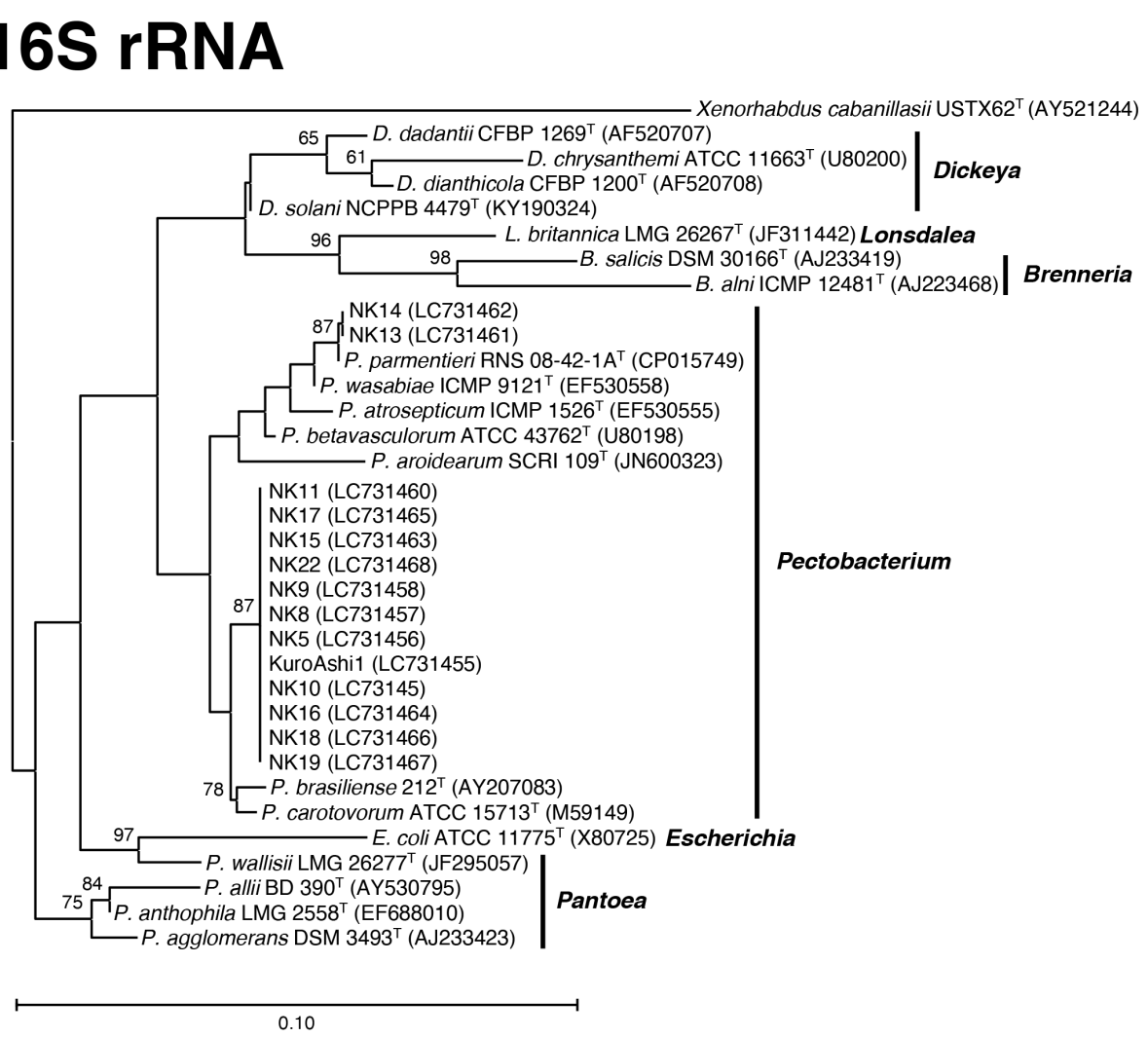

Fig. 2. Maximum likelihood phylogenetic trees based on nucleotide sequences of 16S ribosomal RNA gene of the isolates from blackleg potatoes in Nagasaki Prefecture and type strains of related species. *Xenorhabdus cabanillasii* was used as an outgroup. Accession numbers are shown in parentheses. Bootstrap percentages calculated using 1000 replicates are indicated at the branch. Scale shows substitutions per site.

DISCUSSION

Potatoes are produced by vegetative reproduction, and seed tubers are usually planted. The use of latently infected seed tubers causes severe damage and loss of production. In addition, it causes the spread of pathogens to disease–free areas through seed distribution. Therefore, seed potato production is strictly managed in accordance with the Plant Protection Law in Japan (Kawakami *et al*., 2015). The seed potatoes are propagated in several stages; production of minitubers, propagation in the basic fields, and then in the foundation fields.

Despite these efforts, blackleg disease by *P. brasiliense* was first reported at seed potato fields in Hokkaido Prefecture (Fujimoto *et al*., 2017). The occurrence of potato blackleg disease has increased recently in Japan, and it is pointed out that epidemiological studies of *P. brasiliense* and other pathogens of the disease are needed.

This study revealed that *P. parmentieri* was found only in Shimabara City, and *P. brasiliense* appeared in the three cities in Nagasaki Prefecture. Similarly, these pathogens are the major pathogen causing blackleg in Hokkaido Prefecture (Nakayama *et al*., 2021). The occurrence of blackleg disease of potatoes in Nagasaki Prefecture was sudden and no subsequent outbreaks have been observed. Therefore, it is unlikely that the disease was caused by indigenous pathogens, and it may be reasonable to assume that it was caused by invasive pathogens. *P. brasiliense* and *P. parmentieri* have been reported in various regions of the world; thus, it is unknown at this time where the Nagasaki strains originated and the relationships among the strains. Therefore, diversity analysis using various strains worldwide and further epidemiological studies are also required.

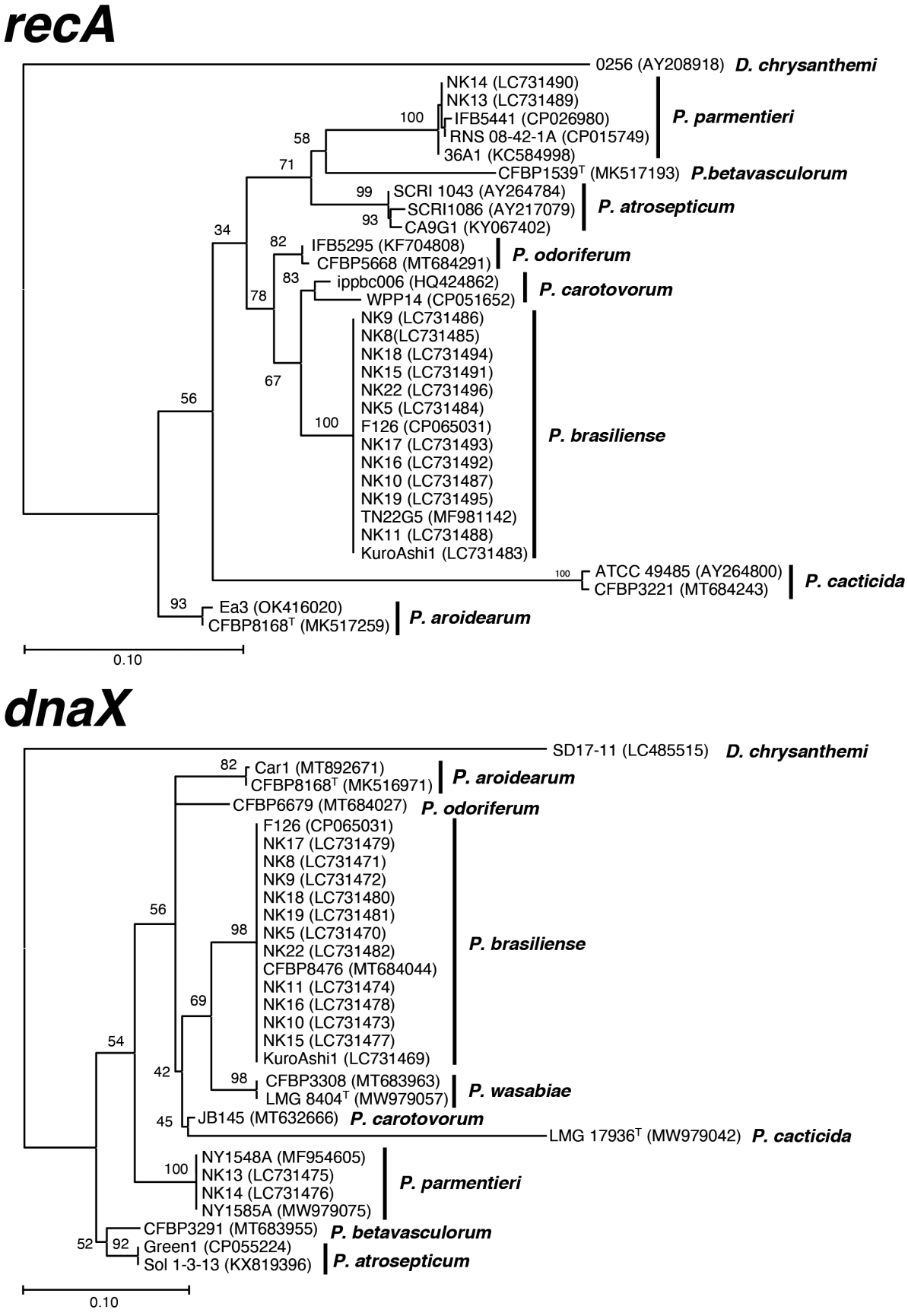

Fig. 3. Maximum likelihood phylogenetic trees based on nucleotide sequences of *recA* and *dnaX* of the isolates from blackleg potatoes in Nagasaki Prefecture and reference strains of *Pectobacterium* species. *Dickeya chrysanthemi* was used as an outgroup. Accession numbers are shown in parentheses. Bootstrap percentages calculated using 1000 replicates are indicated at the branch. Scale shows substitutions per site.

AUTHOR CONTRIBUTIONS

L. Q. Man performed the pathogenicity test and biochemical characteristic tests, analyzed sequences, constructed phylogenetic analyses, and wrote the paper. Y. Suga collected disease samples and performed pathogenicity tests. K. Iiyama designed the study, conducted PCR assays, supervised the work, and wrote the paper. H. Otofuji wrote the paper. K. Tsuchiya performed biochemical and pathogenicity tests, and N. Furuya supervised the work and wrote the paper. All authors assisted in editing the manuscript and approved the final version.

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