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Soft rot of root chicory in Hokkaido and its causal bacteria

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Abstract

In August 2010, bacterial soft rot was found on root chicory (*Cichorium intybus* L. var. *sativum* Bisch.) in Hokkaido, Japan. Severely infected plants in fields were discolored, had wilted foliage, and black necrosis of petioles near the crown. Wilted leaves subsequently collapsed and died, forming a dry, brown or black rosette. The root and crown became partially or wholly soft-rotted. Slimy masses on infected areas of roots, turned dark brown or black. Gram-negative rod-shaped, peritrichously flagellated and facultatively anaerobic bacteria were exclusively isolated from rotted roots, and typical symptoms were reproduced after inoculation with the strains. The bacteria were identified as *Dickeya dianthicola*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Pectobacterium carotovorum* subsp. *odoriferum* based on further bacteriological characterization and the sequence analysis of the malate dehydrogenase gene and 16S rRNA gene. These bacteria should be included with the previously reported *Dickeya* (=*Erwinia*) *chrysanthemi* in Saitama Prefecture, Japan, as causal pathogens of bacterial wilt of chicory.

Keywords: *Dickeya dianthicola* • *Pectobacterium carotovorum* subspecies • Soft rot • Chicory root • Bacterial wilt

Introduction

Chicory (*Cichorium intybus* L. var. *sativum* Bisch.) is a horticultural crop grown for direct consumption as a cooked food and an industrial crop for the extraction of polysaccharide. Root chicory is an alternative sugar crop, which accumulates inulin in its roots. Inulin is a polymer that is widely used in several industrial applications. As an example an oligosaccharide, difructose anhydride III derived from inulin has received increasing interest because of its positive effects on health. Found to stimulate the absorption of calcium in humans, this oligosaccharide has become established as a component in health foods (Kikuchi et al. 2009). At present, inulin is mostly supplied by root chicory, which is mainly grown and processed in European countries such as The Netherlands, France, and Belgium. Because domestic raw materials are in demand for more sustainable and economical production of products, the expansion of root chicory cultivation to Hokkaido in northern Japan is an attractive option. The sowing and harvesting techniques used for sugar beet and potato can be used for root chicory, so no investment in equipment should be necessary, only some minor modifications.

In August 2010, a root rot of chicory was observed during cultivation in experimental fields in Hokkaido. Bacteria were consistently isolated from the rotten tissues and presumed to be the causal agents of the disease. Previously, bacterial wilt of chicory caused by *Erwinia chrysanthemi* was reported in Saitama Prefecture, Japan (Sakai 1995), and we had predicted that this disease would affect chicory in Hokkaido. The purpose of this study was to demonstrate the pathogenicity of the bacteria isolated from the diseased chicory and to confirm the taxonomic positions of these bacteria through bacteriological and genetic characterization.

Materials and methods

Isolation of bacterial strains

Root samples with soft rot were collected from fields at the Ornamental Plants and Vegetables Research Center, Hokkaido Research Organization, Takikawa, and Institute of Nippon Beet Sugar Manufacturing, Obihiro, in August 2010. Bacteria were isolated from the affected tissues as follows. Small pieces of diseased tissue were dissected at the lesion margin and rinsed with sterile distilled water. After drying, about 1 g of tissue was ground in a mortar with 3 mL of sterile distilled water. One loopful of the suspension was streaked on a plate of nutrient agar (NA; Difco, Franklin Lakes, NJ, USA). The plate was incubated at 27°C for 24–48 h, and single colonies were subcultured onto nutrient agar slants. The cultured bacterial cells were suspended in 10% skim milk and stored at –80°C. The skim milk suspensions were also lyophilized for long-term storage. All isolates obtained from chicory in this study were deposited in the National Institute of Agrobiological Sciences (NIAS) Genebank as MAFF (Ministry of Agriculture, Forestry and Fisheries of Japan) accessions listed in Table 1. Eleven reference MAFF isolates of *Erwinia chrysanthemi* (*Dickeya* spp.) from the NIAS Genebank were also used (Table 1).

Pathogenicity tests

Five-week-old chicory (cv. Melci) seedlings were grown in 15-cm-diameter pots with sterile potting substrate (Pot ace; Katakura Chikkarin, Tokyo, Japan) in the greenhouse. All bacterial isolates were grown on King's B agar for 48 h at 27°C and used as inoculum (10^8 cfu/mL). Four-month-old root chicory plants were stabbed with a sterilized needle at a constant depth of 0.5 cm at three locations around the bottom of each root and a drop of bacterial suspensions (10 μL) was placed on the wounds. The inoculated plants were maintained for 3 days in the greenhouse (25-28°C) enclosed in sterile plastic bags. Control plants were inoculated with sterile distilled water as described already. Plants were examined daily for symptoms for 7 days after inoculation. Bacteria were reisolated from inoculated plants on modified CVP medium (SL-CVP, Hélias et al. 2012), and colony identification was confirmed by morphological and biochemical tests.

Phenotype determination

Isolates used in this study were identified by standard bacteriological methods (Dickey 1979; Hauben et al. 2005; Samson et al. 2005; Schaad et al. 2001) using the following tests: gram reaction by KOH solubilization, glucose metabolism in Hugh and Leifson medium, growth at 28°C and 37°C, oxidase activity, and catalase test. Indole production from tryptophan, phosphatase activity, sensitivity to erythromycin (using prepared disk impregnated with 15 μg of erythromycin), and salt tolerance in a nutrient agar medium with 5% NaCl were checked after 48 h of incubation. Pigment production; nitrate reduction; gelatin liquefaction; gas from glucose; reducing substances from sucrose; utilization of α-methyl-D-glucoside, sodium malonate, L-tartrate, and sodium citrate; and acid production from inulin, trehalose, D-xylose, dulcitol, sorbitol, melibiose, raffinose, arabitol, and lactose were also tested. All these tests were incubated at 28°C.

Biovar determination of *Erwinia chrysanthemi* (=*Dickeya* spp.)

The following tests were used for biovar classification (Ngwira and Samson 1990): growth on nutrient agar medium at 39°C, inulin assimilation in phenol red peptone water, and anaerobic hydrolysis of arginine (arginine dihydrolase, ADH) according to Macián et al. (1996). Other carbon sources were tested by acidification/alkalinization on liquid Ayers, Rupp & Johnson's (ARJ) medium (Azegami et al. 1985) with bromothymol blue mixed with 0.3% of the carbohydrate: D-(–)-arabinose, 5-ketogluconate, mannitol, melibiose, raffinose and D-tartrate. These tests were performed using test tubes with added test carbon sources and an additional 50 μL of bacterial suspension at approximately 10^8 cfu/mL in 5 mL of ARJ medium. Incubations were carried out at 25°C for 10–15 days (Palacio-Bielsa et al. 2006).

Host range assays

Potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), Welsh onion (*Allium fistulosum* L.), sweet potato (*Ipomoea batatas* L.), chrysanthemum (*Chrysanthemum morifolium* Ramat.), carnation (*Dianthus caryophyllus* L.), and tomato (*Solanum lycopersicum* L.) were used as test hosts.

Inocula were prepared by suspending 48 h cultures grown on King's B medium in sterilized water (approximately 10^8 cfu/mL). Four potato tubers per experiment were inoculated with the suspensions (10 μL) through wounds by stabbing with a sterilized needle at a constant depth of 0.5 cm. The tubers were incubated in a moist chamber at 28°C for 3 days. Control tubers were inoculated with sterile distilled water, and two independently replicated assays were performed. Sweet potatoes were inoculated using the same method.

Leaf sheaths of five Welsh onion plants per experiment were inoculated with 10 μL of the suspension by inserting a sterile plastic micropipette tip at a constant depth, as described Palacio-Bielsa et al. (2010). They were incubated in a moist chamber at 28°C up to 2 days. Control plants were inoculated with sterile distilled water, and two independently replicated assays were performed. Fruits of the eggplants and stems of the carnation and chrysanthemum were inoculated using the same method.

Stems of 25-day-old tomato plants were injected with 10 μL of the suspension at the third and fourth basipetal axial leaf (Nabhan and Wydra 2011). Control plants were inoculated with sterile distilled water.

PCR identification

For genomic DNA extraction, bacteria were grown in nutrient broth (NB; Difco) in a shaking incubation bath for 16 h at 28°C. DNA was extracted using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified using a BioSpec-nano UV-VIS Spectrophotometer (Shimadzu, Kyoto, Japan). PCR was performed in 50-μL reaction volumes containing 20 ng of target DNA, 0.25 μL of *TaKaRa Ex Taq* (5 units/μL; TaKaRa, Otsu, Japan), 5 μL of 10× *Ex Taq* buffer, 4 μL of dNTP mixture (2.5 mM each), and 1.0 μM of each primer (ADE1/ADE2 primer pair). DNA was amplified using a DNA thermal cycler PC320 (ASTEC, Fukuoka, Japan). Amplified fragments were separated on a 1.5% agarose gel and visualized under UV light following ethidium bromide staining.

The *pel* ADE-specific primers, ADE1 (5′-ATC AGA AAG CCC GCA GCC AGA T-3′) and ADE2 (5′-CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC-3′), amplified a 420-bp pectate lyase-encoding gene fragment from all *E. chrysanthemi* strains tested, but not from any other soft-rot *Erwinia* (Charkowshi 2006). DNA was amplified with 25 cycles at 94°C for 1 min and 72°C for 2 min, with a final elongation step at 72°C for 8 min (Kerr et al. 2009).

Analysis of 16S ribosomal RNA gene and *mdh* gene sequences

The 16S rRNA gene was amplified by PCR in 50-μL reaction volumes containing 20 ng of target DNA, 0.25 μL of *TaKaRa Ex Taq* (5 units/μL; TaKaRa), 5 μL of 10× *Ex Taq* buffer, 4 μL of dNTP mixture (2.5 mM each), 1.0 μM of 16S–F3 (5′-CAG GCC TAA CAC ATG CAA GT-3′), and 1.0 μM of 16S–R3 (5′-GGG CGG WGT GTA CAA GGC-3′) (Shin et al. 2008). DNA amplification was performed using DNA thermal cycler PC320 (ASTEC), with initial denaturation at 94°C for 4 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final elongation step at 72°C for 5 min. Amplified fragments were run on a 1.5% agarose gel and visualized under UV light following ethidium bromide staining and purified using a QIA quick purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified products of tested isolates were cloned with commercial kits (Mighty TA-cloning Kit; Takara) and plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen) before sequencing. Complete DNA was sequenced in both directions (Hokkaido System Science, Sapporo, Japan) using forward and reverse sequencing primers (M13F and M13R).

The conserved sequence in the malate dehydrogenase (*mdh*) gene has been previously shown to be phylogenetically informative for enterobacterial genera (Ma et al. 2007; Palacio-Bielsa et al. 2010). For each bacterial strain tested, partial *mdh* DNA sequences were confirmed by amplifying fragments with the primers *mdh*86F (5′-CCC AGC TTC CTT CAG GTT CAG A-3′) and *mdh*628R (5′-CTG CAT TCT GAA TAC GTT TGG TCA-3′) using the following steps: a hot start at 95°C for 3 min; 30 amplification cycles at 94°C for 0.5 min, 52°C for 0.5 min, and 72°C for 1 min; and a terminal

extension phase at 72°C for 6 min to anneal. The PCR products were then cloned for sequencing described already.

Phylogenetic analysis

The nucleotide sequences were deposited in DDBJ, and A BLAST search in the GenBank sequence database was conducted for closely related sequences. The selected sequences of *mdh* gene of *Dickeya* spp. and *P. carotovorum* subspecies were analyzed using CLUSTALW from the DDBJ Web site (http://clustalw.ddbj.nig.ac.jp/) with distances estimated using Kimura's two parameters (Kimura 1980) model and clustering using the neighbor-joining method (Saitou and Nei 1987). A phylogenetic tree was drawn using MEGA 5.05 software (http://www.megasoftware.net/). A BLAST search in the GenBank sequence database was conducted for closely related sequences of the 16S rRNA gene, and selected sequences of the 16S rRNA gene of *Pectobacterium* spp. were also analyzed using CLUSTALW as described.

Results

Symptoms

In August 2010, soft rot of root chicory was observed in Takikawa (Fig. 1a) and Obihiro. Severely infected plants in the fields were discolored, foliage wilted, and black necrosis developed on petioles near the crown. Wilted leaves subsequently collapsed and died, forming a dry, brown or black rosette. Root and crown became partially or wholly soft-rotted and brown or black lesions were observed on the surface of the roots. On the infected inner area of the root, a slimy mass turned dark brown or black (Fig. 1b). Colonies isolated from diseased roots on nutrient agar had umbonate to coralloid margins at 2–3 days (Fig. 1c).

Pathogenicity to chicory

On plants inoculated with all isolates from chicory, symptom appeared within 7 days after

inoculation (Fig. 2). When roots were inoculated with isolate A-R1 to A-R6 and B-R1 to B-R6, soft rot began as a small, water-soaked lesion from the inoculation sites, which enlarged rapidly in diameter and in depth. Then the sites became soft and mushy, while the surface became discolored and somewhat depressed. When roots were inoculated with MT-5 and MT-7, a translucent and wet rot of the etiolated tissue was associated with the production of a ripe odor. Longitudinal cutting of the root showed that rotting had spread from the inoculated site to most of the tissues and the entire root was soft-rotted. The crown turned into a black and decayed mass. On the upper parts of the crown, the leaves wilted and turned cream-colored or dark brown (Fig. 2b, d). All the MAFF isolates also caused soft rot of the roots. A scab-like lesion on the inoculation area that was darker than the surrounding area formed when the roots were inoculated with isolate B-P2 (Fig. 2c). The texture of the scabbed area ranged from ridged to corky or even lumpy, and the color of the inoculated areas ranged from russet to dark brown or black. No lesions developed either externally or internally on control plants inoculated with sterile distilled water (Fig. 2a).

When bacteria from the symptomatic regions were reisolated on SL-CVP medium, typical pectolytic colonies formed, and no cavity-forming colonies were obtained from the control plants. Based on morphological and biochemical tests, reisolated bacteria were identical to the inoculated bacteria (data not shown).

Biochemical and physiological identification

Strains isolated from diseased chicory were gram-negative, positive for anaerobic growth, growth at 28°C and 37°C, and catalase, but negative for oxidase, and pigment production. The chicory isolates, A-R1 to 6 and B-R1 to 6, were gray cream-yellow, glistening, and mucous to very mucous after 48 h on King's B medium. In addition, they were positive for indole production, phosphatase activity, and gas production from glucose, and sensitive to erythromycin. Acid was not produced from trehalose. Sodium malonate and L-tartrate were utilized, and neither utilized α-methyl-D-glucoside. These biochemical and physiological characteristics indicated that the pathogen belonged to the genus

Dickeya. (Palacio-Bielsa et al. 2006). However, isolates A-R1 to A-R6 were negative for acid production from melibiose and raffinose (Table 2). While isolate B-P2 was similar to *P. carotovorum* subsp. *carotovorum*, isolates MT-5 and MT-7 were closely related to *Pectobacterium carotovorum* subsp. *odoriferum* (Hauben et al. 2005) (Table 2).

All the MAFF isolates were very similar, and their biochemical and physiological tests agreed with those expected for *Dickeya* spp., except that MAFF 301677, 311043, and 311553 were negative for sensitivity to erythromycin, and MAFF 311152 was negative for acid production from melibiose (Table 3).

Classification of *Dickeya* spp. strains into biovars

Isolates from diseased chicory that were identified as *Dickeya* sp. are presented in Table 4. All of them agreed with those expected for former *E. chrysanthemi* biovars 1 or 7, which coincide with *D. dianthicola*. For MAFF isolates, results largely confirmed the biovar determination by Samson et al. (2005), except that MAFF 311041 grew weakly at 39°C. These results indicate that MAFF 302984, 311553, and 311041 are mostly consistent with *E. chrysanthemi* biovar 1 belonging to *D. dianthicola*. The MAFF 311043, 311151, and 311152 isolates completely agreed with *E. chrysanthemi* biovar 5, namely *D. chrysanthemi* bv. *chrysanthemi*. Isolates MAFF 311098, 106634, 301660, 301677, and 301767 were closely related to *E. chrysanthemi* biovar 3 (*Dickeya dadantii* and *Dickeya zeae*).

Host range assays

Host range assays showed some differences in host plant symptoms among the inoculated isolates (Table 5). No lesions were found on noninoculated controls. The chicory isolates, A-R1 to 6 and B-R1 to 6, were mostly pathogenic to potato, eggplant, Welsh onion, sweet potato, chrysanthemum, and carnation. MT-5 and MT-7 were pathogenic to potato (other plants were not tested for these isolates in this study). No symptoms were observed on sweet potato inoculated with A-R5 and B-R3 or on potatoes inoculated with B-R3 and B-R4. However, the chicory isolates (except for B-P2) were not pathogenic to tomato. B-P2 had the broadest host range. After leaves of tomato collapsed and dried,

seedlings wilted at 2 days after inoculation. Control isolates were pathogenic to the host plants from which the strains were isolated: MAFF 311041 and MAFF 301677 were pathogenic to potato; MAFF 311151 and MAFF 311152 could induce soft rot on chicory; MAFF 301767, MAFF 301660, MAFF 106634, MAFF 311043 and MAFF 311553 were pathogenic to eggplant, Welsh onion, sweet potato, chrysanthemum and carnation, respectively. Most of the MAFF isolates caused symptoms in chicory, carnation, potato, and chrysanthemum as well. However, no symptoms were observed on chrysanthemum inoculated with MAFF 301677 or 311098 (Table 5).

PCR identification

The expected 420-bp, PCR-amplified fragments corresponding to the conserved regions of *Dickeya* species' pectate lyases-encoding gene cluster (*pel*ADE) were obtained for all the *Dickeya* spp. isolates (see online Supplemental Figs. 1 and 2).

Phylogenetic analysis

To determine the phylogeny of strains isolated in this study, *mdh* and 16S rRNA genes were cloned and sequenced. Sequences were deposited in the DDBJ database as accession nos. AB744130–AB744155 and AB744158–AB744183. A phylogenetic tree was constructed using the neighbor-joining method and *Aeromonas salmonicida* as an outgroup for the *mdh* gene (Fig. 3). The results of the phylogenetic analyses showed that A-R1 to A-R6, B-R1 to B-R6, and MAFF 311041, 302984, and 311553 were phylogenetically close to *D. dianthicola* CFBP 1200^{T} (GQ891984). Similarly, B-P2 was phylogenetically close to *P. carotovorum* subsp. *carotovorum* ATCC 15713^{T} (FJ895851), while MT-5 and MT-7 were closely related to the strain CFBP 1878^{T} (JF926793) belonging to *P. carotovorum* subsp. *odoriferum*. MAFF isolates 311043, 311151, and 311152 were identical to *D. chrysanthemi* (syn. *E. chrysanthemi* ATCC 11663^{T}) (FJ895839), and MAFF 311098 was similar to *D. zeae* CFBP 2052^{T} (GQ891983). According to Brady et al. (2012), *D. dieffenbachiae* should be reclassified as *D. dadantii* subsp. *dieffenbachiae*. Therefore, MAFF 106634, 301767, 301677, and 301660 were phylogenetically

close to *D. dadantii* subsp. *dadantii* (*D. dadantii* CFBP 1269^T) and *D. dadantii* subsp. *dieffenbachiae* (= *D. dieffenbachiae* CFBP 2051^T).

Phylogenetic analysis based on the 16S rRNA gene showed that MT-5 and MT-7 were grouped together with type strain *P. carotovorum* subsp. *odoriferum* CFBP 1878^T (AF373191) and were distinct from other *Pectobacterium* spp. Strain B-P2 was phylogenetically close to *P. carotovorum* subsp. *carotovorum* DSM 30168^T (AJ233411) (Fig. 4).

Discussion

Fifteen bacterial isolates collected from the root rot of chicory in 2010 were biochemically and genetically identified. *Dickeya dianthicola* and *P. carotovorum* subsp. *carotovorum* were isolated from root of chicory in the Takikawa trial field. Additionally, *P. carotovorum* subsp. *odoriferum* was isolated from root of chicory in the Obihiro trial field. Pathogenicity of these isolates to chicory root was also confirmed in this study.

The pectolytic grouping of the *Enterobacteriaceae* family (formerly the Erwinia soft-rot group) comprised the genera *Pectobacterium* (e.g., *Erwinia carotovora*) and *Dickeya* (e.g., *E. chrysanthemi*) (Nabhan et al. 2012). The bacteria from these two genera had been classified as two separate soft-rot causing pathogens. Because *E. chrysanthemi* (Burkholder et al. 1953) caused disease on a wide variety of plant hosts (Samson et al., 2005; Ma et al., 2007), the species was subdivided into six pathovars (Lelliott and Dickey 1984) and nine biovars (Ngwira and Samson 1990; Samson et al. 1987). Currently, the species was reclassified into the genus *Dickeya* using 16S rRNA, DNA–DNA hybridization and biochemical characterization (Samson et al. 2005). *Dickeya* was divided into six species that correspond to both the pathovar and biovar classifications: *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca*, and *D. zeae* (Bull et al. 2010; Samson et al., 2005). Recently, multilocus sequence analysis data indicated synonymy of *D. dadantii* and *D. dieffenbachiae*; those were reclassified as *D. dadantii* subsp. *dadantii* and *D. dadantii* subsp. *dieffenbachiae*, respectively (Brady et

al. 2012). Moreover, a new *Dickeya* strain, tentatively called *Dickeya solani* (Sławiak et al. 2009) has been isolated that does not fall within those five species. To date, *D. dianthicola* has been found to cause disease on potato, tomato, chicory and artichoke, as well as on ornamental genera such as *Begonia*, *Dahlia, Freesia*, *Hyacinthus*, *Iris*, *Kalanchoe* and *Zantedeschia* (Toth et al. 2011). According to Toth et al. (2011), no potato isolates from outside Europe have been confirmed as *D. dianthicola*, with the exception of one sample from Bangladesh that may have been grown from European seed tubers. However, MAFF 311041, isolated from potato in Hokkaido Japan, was confirmed to be *D. dianthicola* in this study.

Although 16S rRNA sequence analysis is commonly used to identify bacterial species and phylogenetic studies, it was relatively poor at differentiating *Dickeya* species (Sławiak et al. 2009). As expected, the chicory isolates did not cluster separately into each *Dickeya* species (data not shown). Thus, the *mdh* gene was chosen for phylogenetic analysis because it is present in most enterobacteria and its product is involved in diverse aspects of bacterial metabolism. Preliminary analysis suggested that these genes have enough sequence diversity to allow us to reconstruct bacterial phylogenies (Palacio-Bielsa et al. 2010). Consequently, phylogenetic analysis of *Dickeya* species, based on the *mdh* gene, showed that the isolates A-R1 to 6 and B-R1 to 6 were grouped together with type strain *D. dianthicola* CFBP 1200^{T}.

Phylogenetic analysis of the genus *Pectobacterium* spp. strains based on the *mdh* gene and 16S rRNA gene showed that MT-5 and MT-7 grouped together into the type strain *P. carotovorum* subsp. *odoriferum* CFBP 1878^{T} and were distinct from other *Pectobacterium* spp. (Figs. 5 and 6). The host-range of *P. carotovorum* subsp. *odoriferum* is narrow. Since the first reported isolate from chicory in France (Samson et al. 1980), the bacterium has only been found to cause disease on hyacinth, celery, leek, and sugar beet (Gallois et al. 1992; Gardan et al. 2003; Ma et al. 2007). In conclusion, MT-5 and MT-7 should be classified as *P. carotovorum* subsp. *odoriferum*, which was first isolated from slimy rot or odorous soft rot of witloof chicory in France (Gallois et al.1992; Samson et al. 1980).

Previously, "bacterial wilt" (ichô-saikin-byô in Japanese) was suggested and accepted as the disease name indicating soft rot of root in chicory in Saitama Prefecture, Japan, and the pathogen was identified as *E. chrysanthemi* (Sakai 1995). In the present study, isolates MAFF 311151 and MAFF 311152 were identified and confirmed as *D. chrysanthemi* (= *E. chrysanthemi*) based on sequence analysis of the *mdh* gene (Fig. 5). From the description in the report by Sakai (1995) and the pathogenicity test in this study using these isolates, disease symptoms in Saitama were identical to those in the present study. In 2004, *E. chrysanthemi* was isolated from soft-rotted root of chicory in Monbetsu, Hokkaido (Dr. Mitsuo Horita, personal communication). Although further investigation of the distribution of pathogenic bacteria is needed, *D. dianthicola*, *P. carotovorum* subsp. *carotovorum*, and *P. carotovorum* subsp. *odoriferum* should be added as causal pathogens of the bacterial wilt in chicory. To our knowledge, this is the first report of these bacteria causing soft-rot symptoms on root chicory in Japan.

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Figure legends

Fig. 1 Soft rot of root chicory in Hokkaido, Japan. **a** Soft rot of root chicory in trial fields in Takikawa. **b** Infected root area with slimy mass. **c** Colonies of bacterial isolates produced on nutrient agar

Fig. 2 Symptoms of soft rot of chicory observed in pathogenicity tests. **a** Noninoculated check. **b** Inoculated with isolate B-R3. **c** Inoculated with isolate B-P2. **d** Inoculated with isolate MT-5

Fig. 3 Neighbor-joining tree based on concatenated partial *mdh* gene sequences of members of *Dickeya* species and *Pectobacterium carotovorum* subspecies and of members of phylogenetically related species and subspecies. Bootstrap value is marked at the branch. Accession numbers of GenBank sequences are in parentheses. *Aeromonas salmonicida* is included as an outgroup. Type strains are in boldface. *Bar* 0.005 substitutions per site. T, type strain

Fig. 4 Neighbor-joining tree based on 16S rRNA gene sequences of members of *Pectobacterium* species and of members of phylogenetically related bacterial species. Bootstrap value is marked at the branch. Accession numbers of GenBank sequences are in parentheses. *Cronobacter sakazakii* is included as an outgroup. *Bar* 0.005 substitutions per site. T, type strain

Table 1 Isolates used in this study

Isolate	MAFF[a]	Host plant	Location	Year
A-R1	212014	*Cichorium intybus*	Takikawa, Hokkaido, Japan	2010
A-R2	212015	*C. intybus*	Takikawa, Hokkaido, Japan	2010
A-R3	212016	*C. intybus*	Takikawa, Hokkaido, Japan	2010
A-R4	212017	*C. intybus*	Takikawa, Hokkaido, Japan	2010
A-R5	212018	*C. intybus*	Takikawa, Hokkaido, Japan	2010
A-R6	212019	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R1	212021	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R2	212022	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R3	212023	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R4	212024	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R5	212025	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-R6	212026	*C. intybus*	Takikawa, Hokkaido, Japan	2010
B-P2	212020	*C. intybus*	Takikawa, Hokkaido, Japan	2010
MT-5	212027	*C. intybus*	Obihiro, Hokkaido, Japan	2010
MT-7	212028	*C. intybus*	Obihiro, Hokkaido, Japan	2010
Reference isolate[b] of *Erwinia chrysanthemi*				
	311151	*C. intybus*	Urawa, Saitama, Japan	1990
	311152	*C. intybus*	Urawa, Saitama, Japan	1990
	301677	*Solanum tuberosum*	Niigata, Japan	1977
	311041	*S. tuberosum*	Memuro, Hokkaido, Japan	1992
	301767	*S. melongena*	Fukuoka, Japan	1983
	301660	*Allium fistulosum*	Saitama, Japan	1982
	106634	*Ipomoea batatas*	Kushima, Miyazaki, Japan	1996
	311098	*Zea mays*	Uryu, Hokkaido, Japan	1990
	311553	*Dianthus caryophyllus*	Oomura, Nagasaki, Japan	2006
	302984	*Smallanthus sonchifolius*	Zentsuji, Kagawa, Japan	1992
	311043	*Chrysanthemum morifolium*	Naganuma, Hokkaido, Japan	1992

[a] Isolate no. in the National Institute of Agrobiological Sciences (NIAS) Genebank.

[b] Reference MAFF isolates identified as *Erwinia chrysanthemi* were obtained from the NIAS Genebank.

Table 2 Comparison of biochemical and physiological characteristics of bacterial isolates obtained from soft-rotted chicory

Characteristic	Chicory isolates					*Dickeya* spp.[b]	*Pectobacterium carotovorum* subsp. *carotovorum*[c]	*Pectobacterium carotovorum* subsp. *odoriferum*[c]
	A-R1 to 6	B-R1 to 6	B-P2	MT-5	MT-7			
Gram reaction	–[a]	–	–	–	–	–	–	–
Oxidative-fermentative test	F	F	F	F	F	F	F	F
Growth at 28□	+	+	+	+	+	+	+	+
Growth at 37□	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–
Catalase test	+	+	+	+	+	+	+	+
Indole production	+	+	–	–	–	+	–	–
Phosphatase activity	+	+	–	–	–	+	–	–
Sensitivity to erythromycin (15 μg)	+	+	–	–	–	+	–	ND
Growth in 5% NaCl	–	+	+	+	+	V	+	ND
Pigment production	–	–	–	–	–	V	–	–
Nitrate reduction	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+
Gas from glucose	+	+	–	–	–	+	–	–
Reducing substances from sucrose	–	–	–	+	+	–	–	+
Utilization of α-methyl-D-glucoside	–	–	–	+	+	–	–	+
Utilization of carbon sources								
Sodium malonate	+	+	–	–	–	+	–	–
L-tartrate	+	+	–	–	–	V	–	–
Sodium citrate	+	+	+	+	+	+	+	+
Acid production from								
Inulin	–	–	–	–	–	V	–	–
Trehalose	–	–	+	+	+	–	+	+
D-xylose	+	+	+	+	+	+	+	+
Dulcitol	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	+	+	–	–	+
Melibiose	–	+	+	+	+	+	+	+
Raffinose	–	+	+	+	+	+	+	+

Arabitol	–	–	–	+	+	–	–	+
Lactose	+	+	+	+	+	V	+	+

[a] +, all isolates positive; –, all isolates negative; V, isolate instability; F, fermentative; ND, not determined.
[b] According to Samson et al. 2005 and Schaad et al. 2001.
[c] According to Hauben et al. 2005.

Table 3 Comparison of biochemical and physiological characteristics of MAFF isolates[a]

Characteristic	MAFF								*Dickeya* spp.[d]
	106634 301660 311041	301677	301767	302984	311043 311553	311098	311151	311152	
Gram reaction	–[b]	–	–	–	–	–	–	–	–
Oxidative-fermentative test	F	F	F	F	F	F	F	F	F
Growth at 28□	+	+	+	+	+	+	+	+	+
Growth at 37□	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–
Catalase test	+	+	+	+	+	+	+	+	+
Indole production	+	+	+	+	+	+	+	+	+
Phosphatase activity	+	+	+	+	+	+	+	+	+
Sensitivity to erythromycin	+	–	+	+	–	+	+	+	+
Growth in 5% NaCl	+	+	–	–	–	–	–	–	V
Pigment production	–	–	+	–	–	–	–	–	V
Reducing substances from sucrose	–	–	–	–	–	–	–	–	–
Utilization of α-methyl-D-glucoside	–	–	–	–	–	–	–	–	–
Acid production from:									
Sorbitol	–	–	–	–	–	–	–	–	–
Melibiose	+	+	+	+	+	+	+	–	+
Raffinose	+	+	+	+	+	+	+	+	+
Arabitol	–	–	–	–	–	–	–	–	–
Lactose	+	–	+	–	–	+	+	–	V

[a] Reference isolates (MAFF no.) identified as *Erwinia chrysanthemi* were obtained from the National Institute of Agrobiological Science Genebank.

[b] +, positive; –, negative; V, strains instability; F, fermentative.

[c] According to Samson et al. 2005 and Schaad et al. 2001.

Table 4 Biovar identification[a] of *Erwinia chrysanthemi* (=*Dickeya* spp.) isolates

Isolate[b]	Growth at 39℃	Anaerobic hydrolysis of arginine	Inulin assimilation	Carbon sources for acidification or alkalinization						Biovar
				5-Ketogluconate	D-(−)-Arabinose	Mannitol	Melibiose	Raffinose	D-Tartrate	
A-R1 to 6	–[d]	+	+	–	–	+	–	–	+	7
B-R1 to 6	–	+	+	–	–	+	+	+	+	1
MAFF 302984	–	+	+	–	–	+	+	+	+	1
MAFF 311553	–	+	+	–	–	+	+	+	+	1
MAFF 311041	+w	+	+	–	–	+	+	+	+	1
MAFF 311098	+	–	–	–	+	+	+	+	–	3
MAFF 106634	+	–	–	–	+	+	+	+	–	3
MAFF 301660	+	–	–	–	+	+	+	+	–	3
MAFF 301677	+	–	–	–	+	+	+	+	–	3
MAFF 301767	+	–	–	–	+	+	+	+	–	3
MAFF 311043	+	+	+	–	–	+	+	+	–	5
MAFF 311151	+	+	+	–	–	+	+	+	–	5
MAFF 311152	+	+	+	–	–	+	+	+	–	5
Current name[c]										
D. dianthicola	–	+	+	–	–	+	+	+	+	1
D. dadantii and *D. zeae*	+	–	–	+	+	+	+	+	–	3
D. chrysanthemi	+	+	+	–	–	+	+	+	–	5
D. dianthicola	–	+	+	–	–	+	–	–	+	7

[a] According to Ngwira and Samson 1990.

[b] MAFF isolates were obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank.

[c] According to Samson et al. 2005.

[d] +, positive; –, negative; W, weak growth.

Table 5 Host range of bacterial isolates from chicory

Host plant	Isolate from chicory								MAFF[a]										
	A-R1	A-R2	A-R4	A-R5	B-R3	B-R4	B-P2	MT-5	3	3	3	3	1	3	3	3	3	3	3
	A-R3		B-R1					MT-7	1	0	0	0	0	1	1	1	1	0	1
	A-R6								1	1	1	1	6	1	1	1	1	2	1
	B-R2								0	6	7	6	6	0	5	1	1	9	0
	B-R5								4	7	6	6	3	4	5	5	5	8	9
	B-R6								1	7	7	0	4	3	3	1	2	4	8
Potato	+[b]	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
	(r)	(r)	(l)	(l)			(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)
Eggplant	+	+	+	+	+	+	+	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
	(r)	(r)	(r)	(r)	(r)	(l)	(r)				(r)								
Welsh onion	+	+	+	+	+	+	+	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT
	(r)	(ws)	(r)	(r)	(r)	(r)	(r)					(r)							
Sweet potato	+	+	+	−	−	+	+	NT	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT
	(r)	(r)	(r)			(r)	(r)						(r)						
Chrysanthemum	+	+	+	+	+	+	+	NT	+	−	+	+	+	+	+	+	+	+	−
	(bl)	(bl)	(bl)	(bl)	(bl)	(bl)	(bl)		(bl)		(bl)	(bl)	(bl)	(bl)	(bl)	(bl)	(bl)	(bl)	
Carnation	+	+	+	+	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+
	(w)	(w)	(w)	(w)	(w)	(w)	(w)		(w)	(w)	(w)	(w)	(w)	(w)	(w)	(w)	(w)	(w)	(w)
Tomato	−	−	−	−	−	−	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
							(w)												
Chicory	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	(r)	(r)	(r)	(r)	(r)	(r)	(s)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)	(r)

[a] MAFF isolates were obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank.

[b] Typical symptom was shown in the parenthesis. r, rot; l, lesion; ws, water soaked; bl, blight; w, wilt; s, scab; +, virulent; −, avirulent; NT, not tested.

Fig. 1



Fig. 2

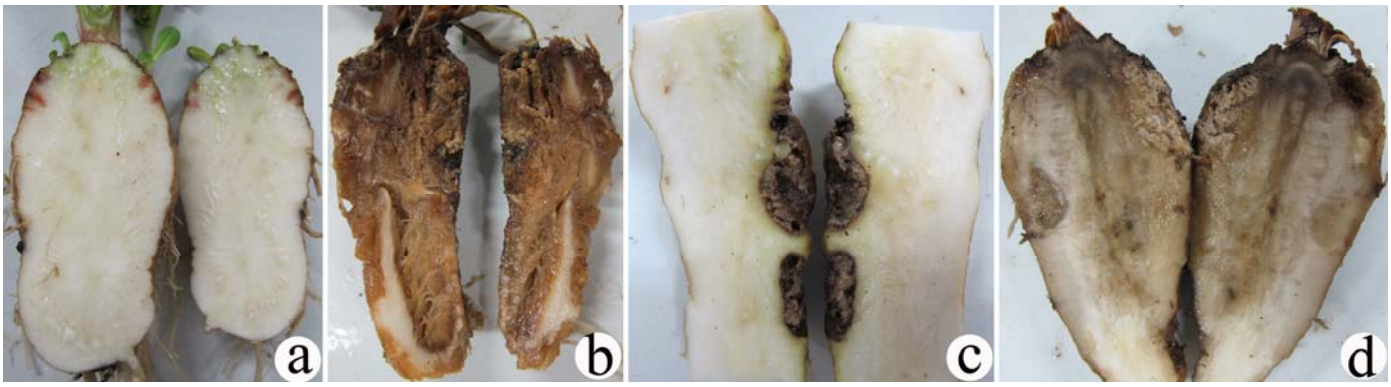


Fig. 3

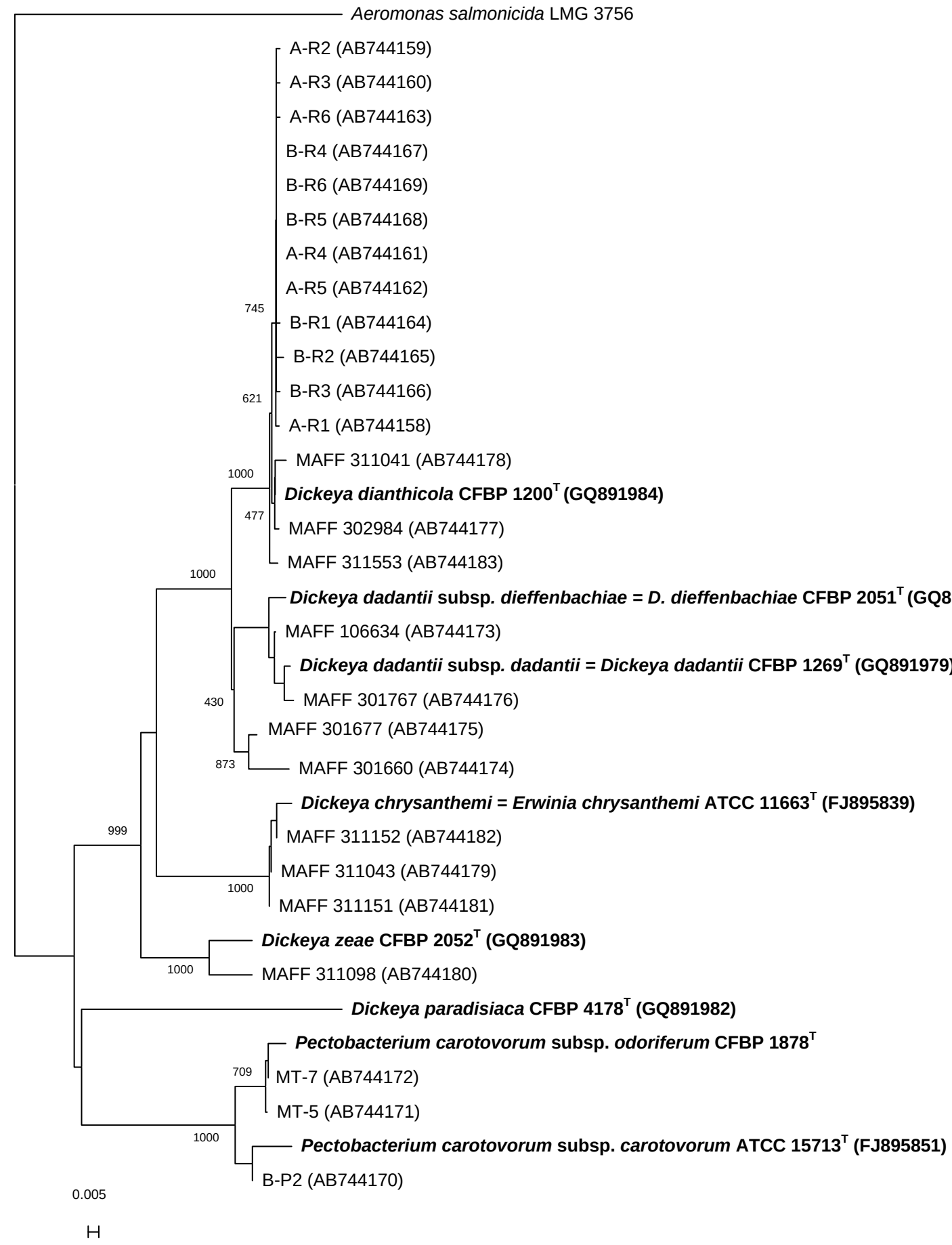
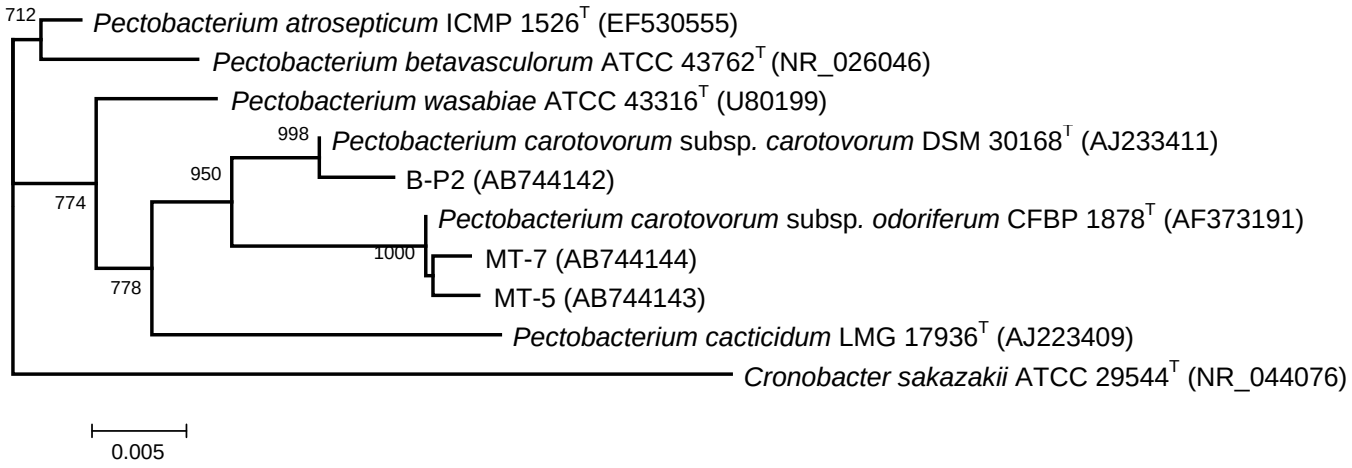

Aeromonas salmonicida LMG 3756
A-R2 (AB744159)
A-R3 (AB744160)
A-R6 (AB744163)
B-R4 (AB744167)
B-R6 (AB744169)
B-R5 (AB744168)
A-R4 (AB744161)
A-R5 (AB744162)
745
B-R1 (AB744164)
B-R2 (AB744165)
621
B-R3 (AB744166)
A-R1 (AB744158)
MAFF 311041 (AB744178)
1000
***Dickeya dianthicola* CFBP 1200^T (GQ891984)**
477
MAFF 302984 (AB744177)
MAFF 311553 (AB744183)
1000
***Dickeya dadantii* subsp. *dieffenbachiae* = *D. dieffenbachiae* CFBP 2051^T (GQ891981)**
MAFF 106634 (AB744173)
***Dickeya dadantii* subsp. *dadantii* = *Dickeya dadantii* CFBP 1269^T (GQ891979)**
430
MAFF 301767 (AB744176)
MAFF 301677 (AB744175)
873
MAFF 301660 (AB744174)
***Dickeya chrysanthemi* = *Erwinia chrysanthemi* ATCC 11663^T (FJ895839)**
999
MAFF 311152 (AB744182)
MAFF 311043 (AB744179)
1000
MAFF 311151 (AB744181)
***Dickeya zeae* CFBP 2052^T (GQ891983)**
1000
MAFF 311098 (AB744180)
***Dickeya paradisiaca* CFBP 4178^T (GQ891982)**
***Pectobacterium carotovorum* subsp. *odoriferum* CFBP 1878^T**
709
MT-7 (AB744172)
MT-5 (AB744171)
1000
***Pectobacterium carotovorum* subsp. *carotovorum* ATCC 15713^T (FJ895851)**
B-P2 (AB744170)
0.005

Fig. 4


712
Pectobacterium atrosepticum ICMP 1526T (EF530555)
Pectobacterium betavasculorum ATCC 43762T (NR_026046)
Pectobacterium wasabiae ATCC 43316T (U80199)
998
Pectobacterium carotovorum subsp. carotovorum DSM 30168T (AJ233411)
950
B-P2 (AB744142)
774
Pectobacterium carotovorum subsp. odoriferum CFBP 1878T (AF373191)
1000
MT-7 (AB744144)
778
MT-5 (AB744143)
Pectobacterium cacticidum LMG 17936T (AJ223409)
Cronobacter sakazakii ATCC 29544T (NR_044076)
0.005

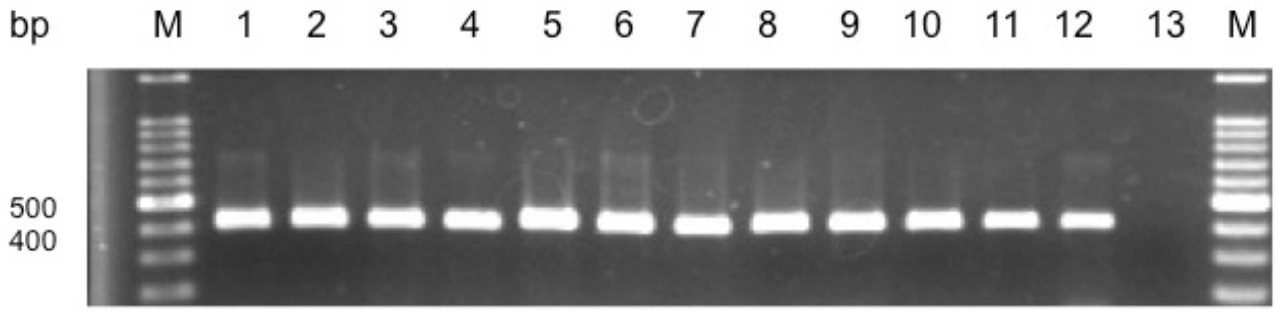
Supplemental Data

Article title:
Soft rot of root chicory in Hokkaido and its causal bacteria

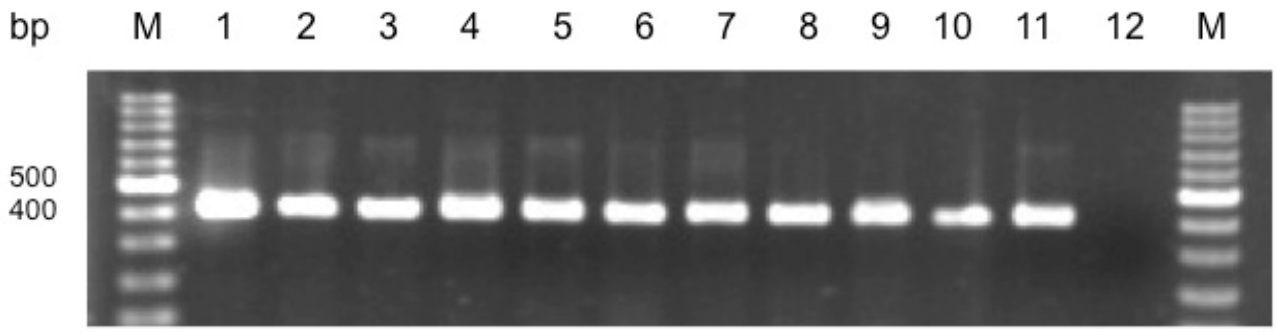
Journal Name:
Journal of General Plant Pathology

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Supplemental Figure 1. PCR amplification of *pel*ADE fragments with primers ADE1 and ADE2. *Lane M* 100-bp DNA ladder marker, *lane 1* A-R1, *lane 2* A-R2, *lane 3* A-R3, *lane 4* A-R4, *lane 5* A-R5, *lane 6* A-R6, *lane 7* B-R1, *lane 8* B-R2, *lane 9* B-R3, *lane 10* B-R4, *lane 11* B-R5, *lane 12* B-R6, *lane 13* PCR negative control (water)



Supplemental Figure 2. PCR amplification of *pel*ADE fragments with primers ADE1 and ADE2. *Lane M* 100-bp DNA ladder maker, *lane 1* MAFF 106634, *lane 2* MAFF 301660, *lane 3* MAFF 301677, *lane 4* MAFF 301767, *lane 5* MAFF 302984, *lane 6* MAFF 311041, *lane 7* MAFF 311043, *lane 8* MAFF 311098, *lane 9* MAFF 311151, *lane 10* MAFF 311152, *lane 11* MAFF 311553, *lane 12* PCR negative control (water)