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Characterization of *Dickeya* strains isolated from potato and river water samples in Finland

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Abstract Plant pathogenic enterobacteria in the genera *Pectobacterium* and *Dickeya* (formerly classified as *Erwinia*) were isolated from diseased potato stems and tubers. The isolated bacteria were identified as *P. atrosepticum*, *P. carotovorum* and pathogens in the genus *Dickeya* with PCR tests. Furthermore, *Dickeya* strains were isolated from river water samples throughout the country. Phylogenetic analysis with 16S-23S rDNA intergenic spacer sequences suggested that the *Dickeya* strains could be divided into three groups, two of which were isolated from potato samples. Phylogenetic analysis with 16S rDNA sequences and growth at 39°C suggested that one of the groups corresponds to *D. dianthicola*, a quarantine pathogen in greenhouse cultivation of ornamentals, while two of the groups did not clearly resemble any of the previously characterised *Dickeya* species. Field trials with the strains indicated that *D. dianthicola*-like strains isolated from river samples caused the highest incidence of rotting and necrosis of potato stems, but some of the *Dickeya* strains isolated from potato samples also caused symptoms. The results showed that although *P. atrosepticum* is still the major cause of blackleg in Finland, virulent *Dickeya* strains were commonly present in potato stocks and rivers. This is the first report suggesting that *Dickeya*, originally known as a pathogen in tropical and warm climates, may cause diseases in potato in northern Europe.

Keywords Blackleg · *Erwinia chrysanthemi* · Soft rot

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Introduction

Contamination of seed tubers with soft-rotting enterobacteria is one of the biggest problems in seed potato production. These bacteria cause blackleg, rotting of potato stems in the field, and soft rot of tubers during

storage. Seed lots are discarded frequently due to blackleg in the field or soft rot in storage. Latent infections in seed tubers often result in blackleg appearing unpredictably in the field. Furthermore, these bacteria also cause severe storage losses for the potato processing industry, especially in large warehouses without refrigeration facilities.

In temperate climates, *Pectobacterium atrosepticum* (former name *Erwinia carotovora* subsp. *atroseptica*; Gardan et al. 2003) has been typically considered to cause both blackleg in the field as well as soft rot during storage, while *Pectobacterium carotovorum* subsp. *carotovorum* (former name *Erwinia carotovora* subsp. *carotovora*; Gardan et al. 2003), has been regarded as the causal agent of soft rot (Pérombelon 2002; Toth et al. 2003). *Dickeya* spp. (former names *E. chrysanthemi* and *P. chrysanthemi*, Samson et al. 2005) have long been recognised as pathogens of ornamental plants particularly in tropical and subtropical climates. In warm climates, *Dickeya* has also been reported to cause both blackleg and soft rot of potato (Pérombelon 2002). *Dickeya* strains have been isolated repeatedly from potatoes during recent decades and the disease has rapidly spread in western Europe during recent years (Hélias et al. unpublished; Dupuis et al. unpublished; Palacio-Bielsa et al. 2006 and references therein). *Dickeya* strains have been isolated from river water in southern Sweden (Persson 1991) and also in many other countries *Dickeya* strains are common in rivers (Cappaert et al. 1988; Cother et al. 1992; Norman et al. 2003).

Variability within the former species *Erwinia chrysanthemi* is large and the strains were divided into several biovars and pathovars (Boccara et al. 1991; Nassar et al. 1996; Avrova et al. 2002). The variability was studied with different methods, such as PCR amplification and sequencing or RFLP of 16S or 16S-23S rDNA intergenic spacer (Toth et al. 2001; Fessehaie et al. 2002), RFLP of recA gene fragments (Waleron et al. 2002) and AFLP fingerprinting (Avrova et al. 2002). Recently, the different strains were reclassified into six genomic species that were placed in the genus *Dickeya* (Samson et al. 2005). The 16S sequences corresponding to these new species are available in public databases.

The aim of this study was to investigate the occurrence, genetic diversity and variation in virulence of different plant pathogenic soft rot enterobacteria in potato samples and in rivers in Finland. Results of this study suggest that *Dickeya* strains are common in river water and can cause blackleg-like symptoms on potato in cool climates. The results indicated that the strains could be divided into three groups, one of which was most likely *D. dianthicola*, while the other groups do not correspond to previously characterised *Dickeya* species.

Materials and methods

Strains, media and culture conditions

Reference strains were obtained from the German Resource Centre for Biological Material (http://www.dsmz.de, strains DSM 4610, DSM 30186, DSM 30170), the National Collection of Plant Pathogenic Bacteria, USA (http://www.ncppb.com, strain NCPPB 402) and the French Collection of Plant Pathogenic Bacteria (CFBP, http://www.angers.inra.fr/cfbp, strains CFBP 1200, CFBP1269, CFBP 1270, CFBP 2051, CFBP 2052, CFBP 3477). *Pectobacterium carotovorum* subsp. *carotovorum* strains SCC1 and SCC3193 have been reported earlier (Pirhonen and Palva 1988). The coding of the Finnish strains isolated in this study shows the identification (Pa for *P. atrosepticum*, Pc for *P. carotovorum* and D. for *Dickeya* sp.) and origin (s indicates that the strain was isolated from a stem, t from a tuber and w from water), the first two numbers tell the year of isolation and the rest is the sample code. Pa s044 and Pa s035-2 are two *P. atrosepticum* strains that were used as technical control strains in virulence tests. The bacterial strains were cultivated on nutrient agar (NA) or in liquid nutrient broth (NB) at 28°C or 39°C. The indole test, which distinguishes *Pectobacterium* from *Dickeya*, CVP-S2 medium (Pérombelon and van der Wolf 2002) and anaerobic test medium (Schaad et al. 2001) have been described earlier.

Isolation of bacterial strains

Collected stem samples were frozen within 1 day after collection to ensure equal handling of all samples collected during several successive days. Bacteria were isolated immediately after collection from tuber and water samples. Isolation of bacteria from samples was carried out according to Pérombelon and van der Wolf (2002). Briefly, small amounts of stem and tuber

samples from the margin of healthy and diseased tissue were macerated in sterile water and a loopful of the liquid or 100 µl of 10-fold serial dilutions were plated on CVP-S2 plates. Water samples were collected from main streams of the rivers (when possible) at 10 cm below the surface directly into sterile plastic containers, and 150 ml of the water was filtered (Whatman number 91) and centrifuged for 30 min (9,000 rpm). After centrifugation, most of the water was removed by pipetting, while 100 µl of the remaining water was plated on CVP-S2 plates and incubated at 27°C for 48 h. Bacteria forming characteristic cavities on CVP-S2 plates were purified, if needed, by repeated sub-culturing on CVP-S2 media. Single cavity-forming colonies were transferred onto NA and grown at 27°C and their ability to grow anaerobically was tested.

PCR

The isolated bacteria were cultured on NA medium at 27°C for 48 h and the colonies were scraped using an L-rod and suspended in 500 µl sterile water. Suspensions were boiled in a water bath for 10 min and frozen. Samples were diluted 1/10 and 1/100 before PCR analysis. Published primers for *P. atrosepticum* (DeBoer and Ward 1995) and *Dickeya* (Nassar et al. 1996) and unpublished *P. carotovorum* primers (MTT Agrifood Research Finland) were used to identify the strains.

Amplification and sequencing of 16S-23S and 16S rDNA sequences

For genomic DNA extraction, bacteria were grown in NB liquid medium at 27°C overnight. DNA was extracted using a commercial extraction kit (DNeasy Tissue Kit, Qiagen, Hilden, Germany) or purified from SDS-lysed cells with phenol-chloroform extraction followed by DNA precipitation with ethanol. The universal DNA primers (1114f and L1r) were used to amplify the 16S-23S rDNA intergenic region and the PCR reactions were performed as described earlier (Fessehaie et al. 2002). PCR fragments for sequencing were prepared from smaller products (~800 bp for *Dickeya* and ~900 bp for the others) by purification of the bands with commercial purification kits (QIAquick Gel Extraction Kit, Qiagen or E.Z.N.A. Gel Extraction Kit, Omega Bio-Tek, Doraville, GA, USA). The PCR products of some of the strains were cloned with commercial kits (Zero Blunt TOPO PCR Cloning Kit, InVitrogen, Carlsbad, CA, USA) and plasmid purified (Plasmid Mini Kit, Qiagen) before sequencing. Complete DNA sequencing (primers of 1491f and L1r for purified gel products and M13F and M13R for cloned samples) was carried out in both directions with a MegaBACE™ DNA Analysis System (Amersham Biosciences) using the DYEnamic™ ET dye terminator cycle sequencing. The post-reaction clean up was carried out by ethanol precipitation. After sequencing, both sequence strands were assembled into a single contig using CodonCode Aligner 1.4.6 (CodonCode Corporation, Dedham, MA, USA) or Sequencer 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) analysis programmes. If needed, alignments were corrected manually. The 16S rDNA sequences were obtained from a service laboratory (University of Turku, Finland, http://www.med.utu.fi/ylab/inenglish.html) specializing in bacterial identification with 16S sequences.

Phylogenetic analyses

For phylogenetic analyses, the 16S-23S and 16S sequences were aligned using Muscle (Edgar 2004) with default parameters. Phylogenetic trees were built up using MrBayes 3.1.1 programme (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) applying the HKY evolutionary model (Hasegawa et al. 1985). The MrBayes was run for 500,000 generations. The first 125,000 generations were discarded as a burn-in phase, and finally every 100th generation was sampled for building up the phylogenetic tree.

Fatty acid analysis

Fatty acid (FAME) analysis of some of the strains was carried out using the Sherlock Microbial Identification System (Midi Inc., Newark, DE, USA) and the TSBA50 library (V 5.00) at the Department of Applied Chemistry and Microbiology, University of Helsinki, Finland.

Virulence assays

Bacterial suspensions for inoculations were prepared by culturing 15 of the newly isolated *Dickeya* strains

and a virulent *P. atrosepticum* strain on NA plates at 27°C for 48 h. Bacteria were scraped using an L-rod and placed in sterile water, and the optical density of the suspension was adjusted to an absorbance of 0.2 at 600 nm using a GeneQuant *pro* RNA/DNA Calculator (Amersham Pharmacia Biotech). Bacterial numbers in the suspensions were verified by dilution plating and counting of bacterial colonies. An OD value of 0.2 was equivalent to ~5×10^8–10^9 cfu ml^{-1}, depending on the strain. Positive (virulent *P. atrosepticum* strain) and negative (no treatment or water) controls were included in tuber and stem tests.

For tuber maceration tests, tubers of cv. Bintje were washed in water and air dried. Ten tubers per strain were pierced at both ends with a micropipette tip (1,000 μl). One end was inoculated with 100 μl of bacterial suspension and then covered with white Vaseline and the other end of tuber was a negative control and was covered only with Vaseline. Tubers were incubated for 4 days at 22°C in 10 moist boxes covered by plastic bags, each box containing in random order 16 tubers inoculated with one of the *Dickeya* or positive control strains. The trial was repeated three times. To assess the amount of macerated tissue, tubers were weighed before and after removal of rotted tissue.

For the stem tests, tubers (cv. Bintje) were planted in 3.5 l pots and grown in a glasshouse until the stems were 30–40 cm. A small hole was made in the stem 5–10 cm above the soil line with a micropipette tip and 10 μl bacterial suspension was introduced into the hole and covered with Vaseline. One or two stems per plant and five replica plants per strain were inoculated. The inoculated plants were grown under controlled temperature conditions of 21°C (night) and 23°C (day) and with 60–95% relative humidity. The light period was 16 h. Plants were arranged in a randomized complete block design with five blocks, that is, each block contained in random order plants inoculated with one of the *Dickeya* or positive control strains. The presence and the length of rotten lesions were recorded during a 3-week period. The trial was repeated twice and statistical analysis was performed on the values of the last scoring.

Field test

Bacterial suspension for tuber inoculation was prepared as in the virulence assays. Seventy-seven tubers of cv. Nicola per each *Dickeya* strain were vacuum infiltrated (−0.7 bar) for 5 min in 5 l of bacterial suspension containing ~5×10^5–10^6 cfu ml^{-1}. The cv. Nicola (certified seed A) was chosen for this experimental because of its low *P. atrosepticum* amount measured from stolon end samples by real-time PCR (Finnzymes Ltd, Espoo, Finland, data not shown). Positive (*P. atrosepticum*) and negative (water) controls were included in the test.

Inoculated tubers were planted manually at the end of May 2006 in random order. Fertilizer was applied according to standard recommendations. Herbicide sprays were applied twice and fungicide against late blight five times and the experiment was irrigated five times during the summer because it was an exceptionally dry season. The health and appearance of the individual plants was assessed and recorded weekly during July and August.

Statistical analysis

The statistical analyses for the tuber and stem virulence tests were constructed following the randomised complete block design with 10 and 5 blocks (boxes), respectively. The mixed models were fitted separately for tuber and stem data with strain, trial and their interaction as fixed effects. In the tuber analysis, tuber nested in the trial was taken as a random effect. Correspondingly, the random effects for the stem analysis were stem nested in the trial and stem nested in the trial and strain (Littell et al. 1996). The analyses for tuber and stem virulence tests were repeated by replacing the original strains by the three *Dickeya* groups (Fig. 3). Because the assumption of homogeneity of variance was not fulfilled, a logarithmic transformation for the percentage of macerated tuber tissues (g) and an angular transformation (arcsin (√stem) for the stem lesion (cm) were carried out before the analyses. The significance levels of the pairwise comparisons between the strain groups were adjusted by the Tukey–Kramer multiple comparison method. Estimated means and 95% confidence intervals are presented. The relationship between the tuber maceration and stem lesion formation was studied by the Pearson correlation coefficient. The logistic regression analysis was carried out for the field trial to compare the ability of the *Dickeya* groups to cause symptoms. P values <0.05 were regarded as statistically significant. All the analyses were performed

using the MIXED procedure (SAS 9.1, SAS Institute Inc., Cary, NC, USA).

Results

Isolation of *Pectobacterium* and *Dickeya* species from potato and river samples

In total, 85 potato stem samples containing blackleg or aerial stem rot were collected from 39 fields of 23 potato growers during the summers of 2004 and 2005. Rotted tubers were gathered from 23 potato lots stored in 15 warehouses during the same years. Furthermore, 36 river water samples were collected between June and August from rivers along the western and southern coastal regions of Finland. Pectolytic bacteria that scored positive in the anaerobic test were successfully isolated from 64 stem samples, 21 tuber samples and 11 river samples and identified as *P. atrosepticum*, *P. carotovorum* and *Dickeya* with PCR tests. In total, more than 400 strains were identified as soft rot bacteria in the PCR tests. Because several samples were collected from each lot and several strains per sample were isolated, only one to three strains from each contaminated lot were chosen for further characterisation by sequencing. These strains included 27 *P. atrosepticum*, 20 *P. carotovorum* and 18 *Dickeya* strains originating from diseased stems, and 12 *P. atrosepticum*, 13 *P. carotovorum* and 2 *Dickeya* strains isolated from tuber samples. The *Dickeya* strains correspond to samples collected from eight individual growers and from all the other samples only *P. atrosepticum* or *P. carotovorum* were identified. Many of the *P. atrosepticum* or *P. carotovorum* strains collected during 2005 were not characterised further. More than 50 soft rot enterobacteria strains were isolated from 11 river samples, and 24 of them were chosen for further characterisation. All strains obtained from water samples were identified as *Dickeya* spp., except for one *P. carotovorum* strain isolated in 2005. Furthermore, PCR tests performed directly on diluted CVP media with mixed cultures grown from water samples, showed that eight additional rivers contained *P. carotovorum*, *Dickeya* spp. or both.

The isolation of bacteria in the *Dickeya* genus was verified with the indole test, which showed that the isolated *Dickeya* strains and all the *Dickeya* type strains (Table 1) were positive, whereas all the other strains were negative (data not shown). These results demonstrated that contrary to what has been believed, *Dickeya* spp. are associated with blackleg and soft rot disease of potatoes and are common in river water in Finland.

Phylogenetic analysis with 16S-23S and 16S rDNA sequences

In order to examine relationships among isolated strains, the 16S-23S intergenic spacer regions of 116 strains were amplified and sequenced. At least two

Table 1 Reference strains and their origin

Strain	Species	Origin	Source of the strain and information
DSM 30186	*P. atrosepticum*	Potato	The German Resource Centre for Biological Material
SCRI 1039	*P. atrosepticum*	Potato	Scottish Crop Research Institute
SCRI 1043	*P. atrosepticum*	Potato	Scottish Crop Research Institute
DSM 30170	*P. carotovorum*	Potato	The German Resource Centre for Biological Material
SCC3193	*P. carotovorum*	Potato	Prof. Tapio Palva, University of Helsinki
SCC1	*P. carotovorum*	Potato	Prof. Tapio Palva, University of Helsinki
DSM 4610[a]	*D. chrysanthemi*	*Chrysanthemum morifolium*	The German Resource Centre for Biological Material
NCPPB 402[a]	*D. chrysanthemi*	*Chrysanthemum morifolium*	National Collection of Plant Pathogenic Bacteria
CFBP 1200	*D. dianthicola*	*Dianthus caryophyllus*	French collection of plant pathogenic bacteria
CFBP 1269	*D. dadanti*	*Pelargonium capitatum*	French collection of plant pathogenic bacteria
CFBP 1270	*D. chrysanthemi*	*Parthenium argentatum*	French collection of plant pathogenic bacteria
CFBP 2051	*D. dieffenbachiae*	*Dieffenbachia* sp.	French collection of plant pathogenic bacteria
CFBP 2052	*D. zeae*	*Zea mays*	French collection of plant pathogenic bacteria
CFBP 3477	*D. paradisiaca*	*Musa paradiciaca*	French collection of plant pathogenic bacteria

[a] The strains DSM 4610 and NCPPB 402 were later found to be identical.

clear PCR products from each *Dickeya* strain were obtained when the 16S-23S rDNA was amplified. Some isolates from water had additional bands. The presence of several bands has been observed earlier by Fessehaie et al. (2002), who used the smallest band for phylogenetic analysis of *Pectobacteria*. The smallest 16S-23S PCR fragments, having the size of ~800 bp for *Dickeya* and ~900 bp for the others, of the isolated strains were sequenced, and from one sample an additional band was sequenced and seen to be almost identical with the sequence from the smallest band (data not shown). Blast analysis with the sequences confirmed the PCR identification of the isolates (data not shown) and showed that all the *Dickeya* strains isolated from the same field or water sample had identical 16S-23S sequences (strains D s0431-2 and D s0431-4, D s0432-1 and D s0432-2, s053-3 and s053-6, Fig. 1). Because the presence of *P. atrosepticum* and *P. carotovorum* in potato samples was expected, only *Dickeya* strains were characterised further.

The multiple sequence alignment with the 16S-23S sequences from Finnish strains and published *Dickeya* sequences showed three groups (Fig. 1, where only the most variable area is shown). The strains with closest resemblance to the published *Dickeya* sequences originated from potato and two river samples from southern Finland (group I in Fig. 1). The sequences of these strains were identical (except one base change in the strain D t042, data not shown). The second group contained strains isolated from one potato sample and from three rivers (group II in Fig. 1). Group III in the alignment consisted of strains having an 11 bp insert not seen in the other strains. These strains were isolated from several river samples but not from potatoes.

In phylogenetic analysis with 16S-23S sequences, the *Dickeya* strains were clearly separated from *Pectobacteria*, and the three *Dickeya* groups classified into the three divergent groups I, II and III, the clade credibilities of the groups varying between 0.75 and 1.00 (Fig. 2). From some of the strains partial 16S sequences were also determined and compared in phylogenetic analysis with the published 16S sequences of characterised *Dickeya* species (Samson et al. 2005). Also in the 16S tree the isolated strains were separated into the same three groups (Fig. 3). Group I, primarily isolated from potatoes, did not cluster together with the previously characterised *Dickeya* species in the 16S tree. Group III, isolated only from water samples, grouped closest to *D. dieffenbachia*, but the clade probability of 1.00 suggested that it was different. Group II clustered together with *D. dianthicola*. According to the classification of Samson et al. (2005), only *D. dianthicola* strains are able to grow at 39°C. When the growth of the published reference strains for different *Dickeya* species (Table 1) and the isolated *Dickeya* strains was tested on NA at 39°C, only

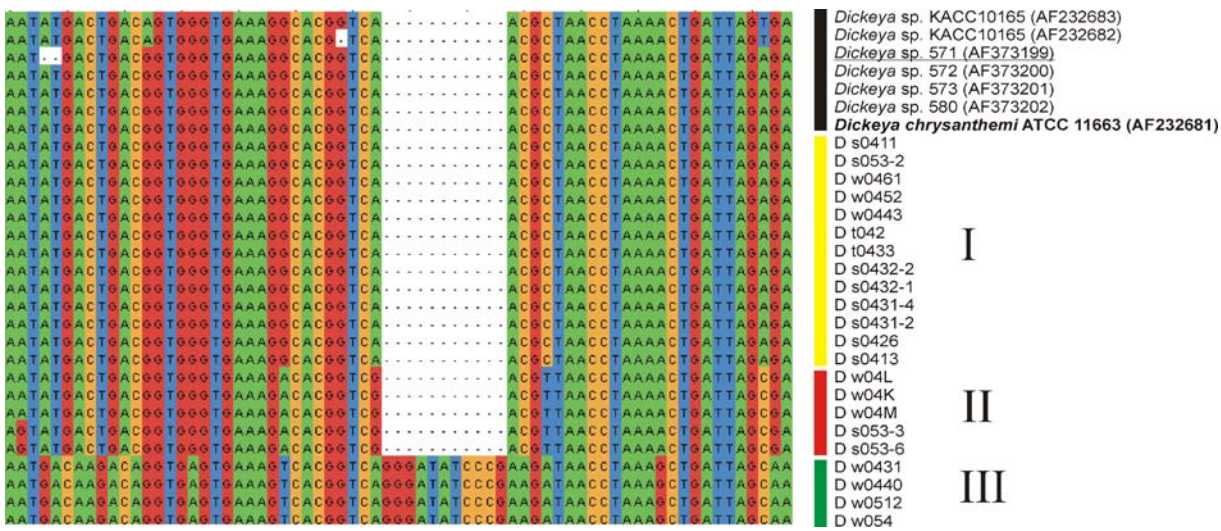

Fig. 1 Multiple sequence alignment of the most variable part of 16S-23S rDNA intergenic spacer region of *Dickeya* strains. Reference sequences obtained from GenBank are marked with a black bar, the *D. chrysanthemi* type strain is marked with *bold* and the reference strain obtained from potato is *underlined*. The origin of the Finnish strains is included in their coding; *s* indicates that a strain was isolated from a stem, *t* from a tuber and *w* from water and the three groups are indicated with *bars* with *different colours* and *roman numbers*

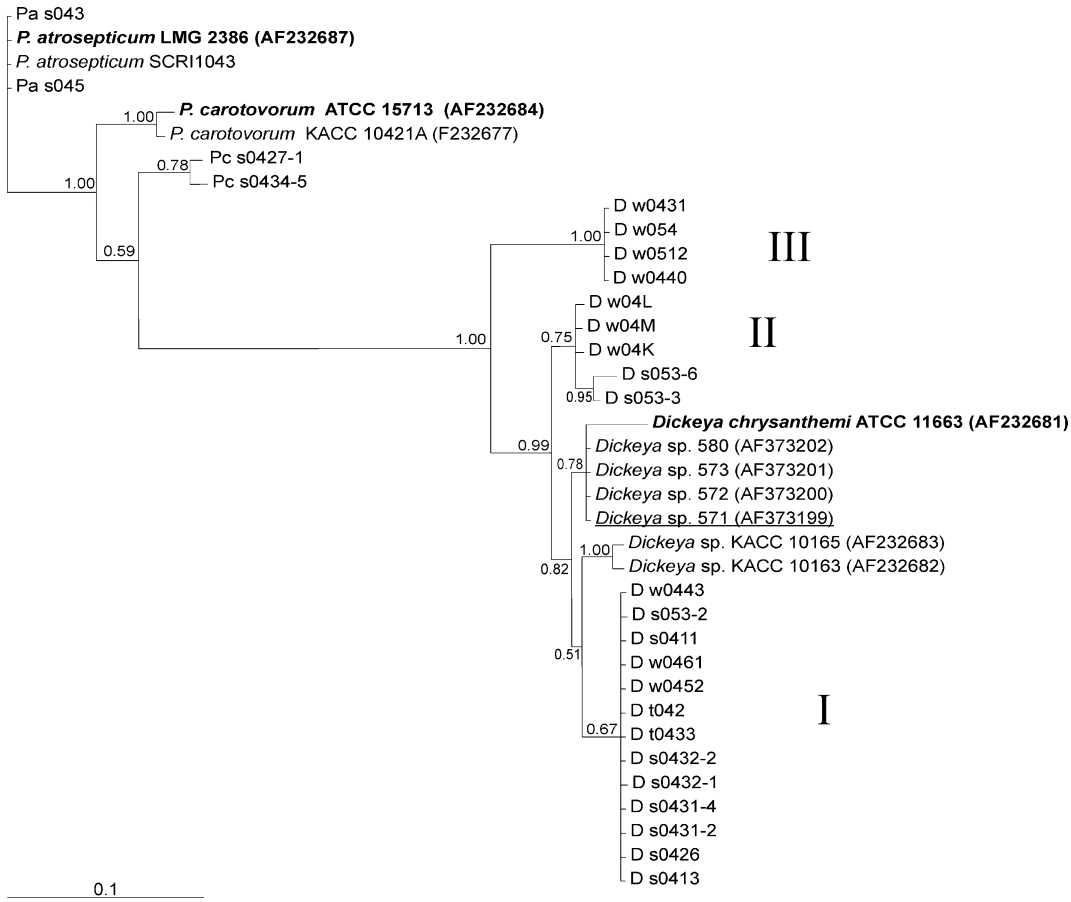

Fig. 2 Phylogenetic tree based on 16S-23S rDNA intergenic spacer sequences of *Dickeya* strains. *Pectobacterium atrosepticum* and *P. carotovorum* are shown as out groups and these groups contain sequences of strains isolated in this study and reference sequences obtained from GenBank. Type strains are marked with *bold* and the *Dickeya* reference strain obtained from potato is *underlined*. *Dickeya* groups are numbered as in the Fig. 1. The lengths of the branches are proportional to the nucleotide changes

the *D. dianthicola* strain CFBP 1200 and the Finnish strains w04K, w04M, w04L, s053-3 and s053-6 were not able to grow to single colonies, suggesting that the strains in the group II were *D. dianthicola*.

Fatty acid analysis

Fatty acid analysis was performed on some *Dickeya* isolates to confirm the identification of the strains as *Dickeya* and to determine their biotype. According to the fatty acid profiles, all the strains were *Dickeya*, and the strains D w04M, D w04K and D s0432-1 resembled most the biotype II, D w0443 the biotype III and the strains D w0431 and D w0440 biotype IV. Nevertheless, for most of these strains the fatty acid analysis could not reliably separate the different biotypes so the remaining strains were not analysed.

Geographical distribution of the isolated strains

Strains in the three *Dickeya* groups were present in rivers in different geographic regions (Fig. 4). *Dickeya dianthicola*-like strains were present in three rivers in the eastern part of the country, group II was isolated from two rivers close to Helsinki and the water-specific strains were present in many rivers. The potato samples from which *Dickeya* strains were isolated were collected from different parts of the country (Fig. 4).

Stem and tuber virulence assays

Virulence of some strains in each identified *Dickeya* group was assayed in stem and tuber tests to identify differences within and between the groups (Fig. 5). Recently isolated virulent *P. atrosepticum* strain, Pa

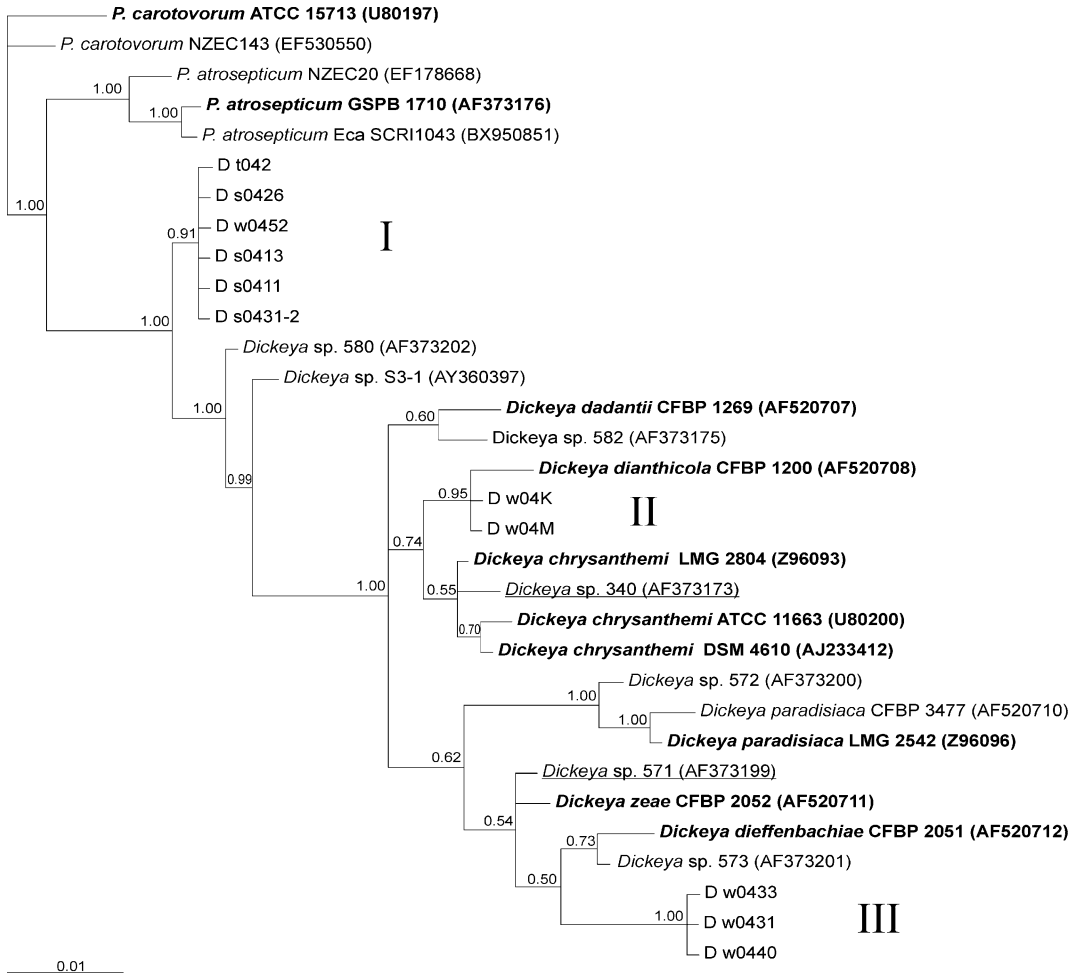

Fig. 3 Phylogenetic tree based on 16S rDNA sequences of *Dickeya* species and strains. *Pectobacterium atrosepticum* and *P. carotovorum* sequences from GenBank are shown as out groups. Type strains are marked with *bold* and the *Dickeya* reference sequences obtained from strains isolated from potato are *underlined*. The three *D. chrysanthemi* sequences shown in the figure are from the same strain. *Dickeya* groups are numbered as in Figs. 1 and 2. The lengths of the branches are proportional to the nucleotide changes

s044, was used as a positive and water treatment as negative control in both assays. Most of the inoculated plants showed typical symptoms of stem and tuber rot, but some showed either no symptoms or symptoms that did not develop further. Significant differences were found in the virulence of the strains in both tuber and stem inoculation tests ($P<0.0001$). Variation was highest among the strains in group I (Fig. 5). Variation between the two repeats of stem assays and among the three of tuber assays was also significant ($P<0.0001$ and $P=0.005$, respectively). No significant interaction between the strain and trial was seen in the stem assays, whereas this was seen in the tuber assay ($P<0.0001$) due to variable results obtained with four strains (D w0431 and group II strains: D w04L, D w04M and D w04K). In spite of the interaction between the strain and trial in the tuber assay, the results of the tuber assay (Fig. 5b) were very similar to the results of the stem assay, and there was a strong positive correlation ($r=0.85$) between them.

According to the Tukey–Kramer test, group I differed significantly from groups II and III ($P<0.001$) in the stem virulence tests, whereas there was no difference between groups II and III. In tuber assays, groups I and III differed in all three assays ($P<0.02$), but group II gave unclear results as it was similar in different trials either to group I or group III.

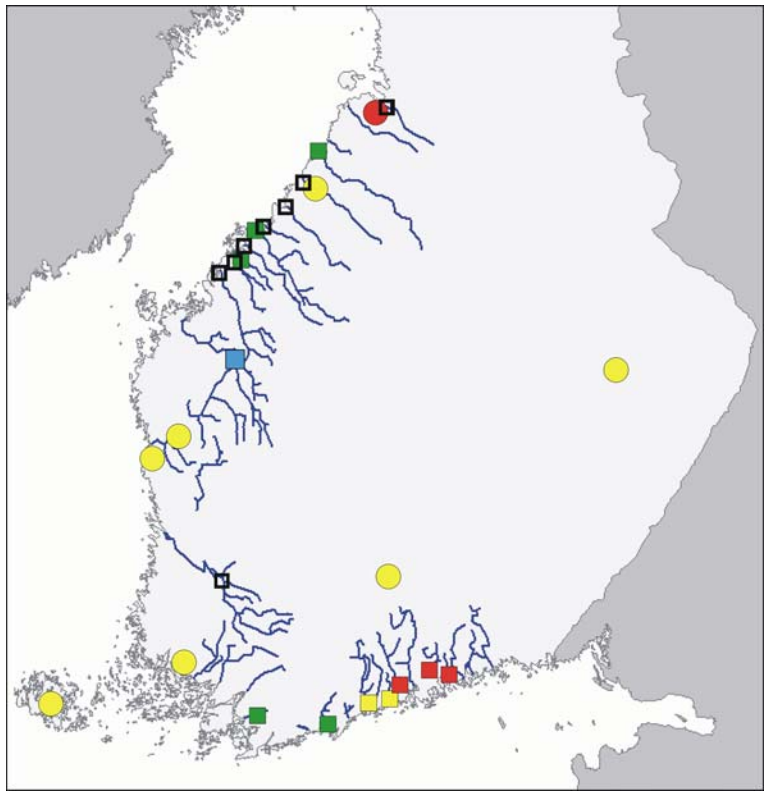

Fig. 4 Geographical distribution of the three groups of *Dickeya* in Finland. *Coloured squares* indicate water samples and *circles* potato samples. Different *Dickeya* groups are shown by the *same colours* as in Fig. 1 (group I with *yellow*, group II with *red* and group III with *green*). The *blue square* represents the single isolated *P. carotovorum* strain from river samples and *empty squares* indicate water samples identified positive for *Dickeya* and/or *P. carotovorum* with PCR without strain isolation

Field trial with the *Dickeya* strains

To study the ability of the isolated *Dickeya* strains to cause disease in the field, a field trial with 10 Finnish *Dickeya* strains was conducted during summer 2006. *Pectobacterium atrosepticum* strain Pa s035-2, that caused symptoms in a pilot test the previous year, was used as positive control for the field trial. Six strains with different levels of virulence in the stem and tuber assays were chosen from group I for the field assay along with two strains from group II and two from group III. Non-emergence or blackleg-like symptoms and necrosis of the central pith of the stem were found within all the groups (Fig. 6). In the beginning of the growing season in July the symptoms resembled blackleg caused by *P. atrosepticum* (Fig. 6a), whereas later during the growing season more necrosis of the pith was observed (Fig. 6b). *Dickeya dianthicola*-like strains in group II caused symptoms in the majority of the inoculated plants (Table 2). Four strains in group I rotted approximately 25% of the inoculated plants, whereas two strains seemed relatively non-virulent. The *Dickeya* strains in group III and the positive and negative controls rotted only a few plants. Logistic regression analysis on the plants showing symptoms (non-emergence, blackleg or necrosis) indicated that the virulence of the groups differed significantly ($P<0.0001$), and the virulence of the group II was significantly higher ($P<0.0001$) compared to all the other groups, including *P. atrosepticum* used as a positive control. Group I did not differ from the positive control, but differed significantly ($P=0.0003$) from the water treatment, whereas group III did not. In conclusion, statistically, the isolates in the *D. dianthicola*-like group were more virulent than the other strains, the virulence of group I was similar to virulence of *P. atrosepticum* and group III did not cause disease in the field trial.

Discussion

This survey was initiated, because representatives of Finnish potato processing and seed potato companies claimed that losses due to blackleg and soft rot of potatoes had increased during the last years. The results of the survey revealed that *Dickeya* strains were isolated from samples collected from the fields

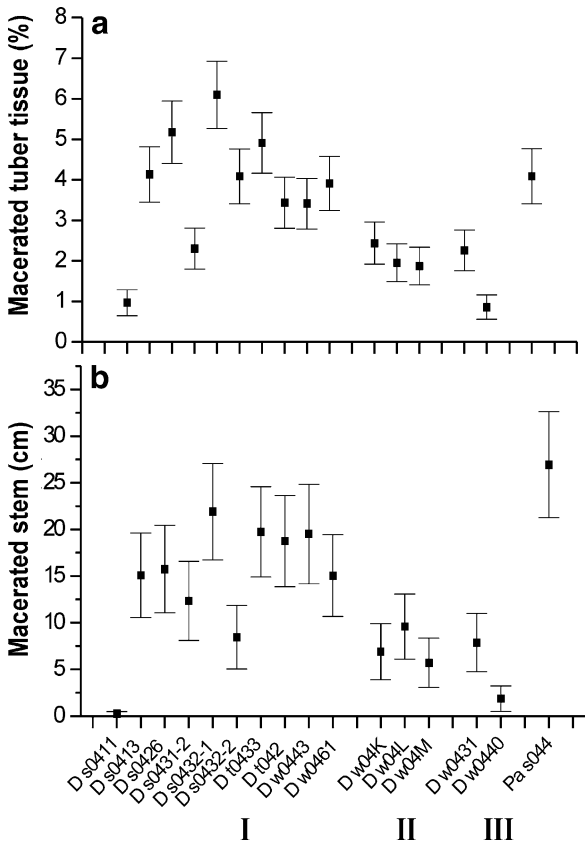

Fig. 5 Estimated means and 95% confidence limits of **a** macerated tuber tissues (g) and **b** length of disease lesions in stems (cm) inoculated with 15 *Dickeya* strains and one control positive strain (*P. atrosepticum* s044). The 95% confidence intervals express the range in which macerated tuber and stem lesion values occur with P=0.95

of 15% of the potato growers, many having fields with a high incidence of rotten plants. The results of the present study support the view that the importance of *Dickeya* spp. as potato pathogens has increased during recent years in Europe. *Dickeya* spp. have been reported to cause 14–80% of the blackleg in France (Hélias et al. 2004) and 38% in Belgium (Dupuis et al. 2005). According to our results, it is no longer restricted to countries in western and central Europe but has spread north, possibly due to climate change and increased import of agricultural products, and may be the cause of increased incidences of blackleg and soft rot in Finland. The results also support the theory that temperate *Dickeya* strains exist, as has been suggested earlier (Janse and Ruissen 1988; Pérombelon 2002).

In addition to strains isolated from stem and tuber samples, *Dickeya* strains were also isolated from several water samples taken from rivers along the coast. In earlier studies *P. carotovorum* strains have been the most common soft rot bacterium in water samples, and the reason for this has been suggested to be its wide host range and good survival in different environments (Pérombelon and Hyman 1987, and references therein). However, *Dickeya* strains have also been isolated repeatedly (Cappaert et al. 1988; Persson 1991; Cother et al. 1992; Norman et al. 2003). Our results suggest that in Finland *Dickeya* strains may be more common than *P. carotovorum* in rivers, possibly due to increased water temperatures during summers.

The Finnish *Dickeya* strains were classified into three groups based on a phylogenetic tree constructed from the sequences of the 16S-23S rDNA intergenic spacer region. The 16S-23S rDNA intergenic spacer region was used for phylogenetic analysis and identification of the strains, because it has been shown to work well for the characterisation of soft rot enterobacteria (Fessehaie et al. 2002). However, because 16S rDNA sequences can be used to classify *Dickeya* strains into six species (Samson et al. 2005),

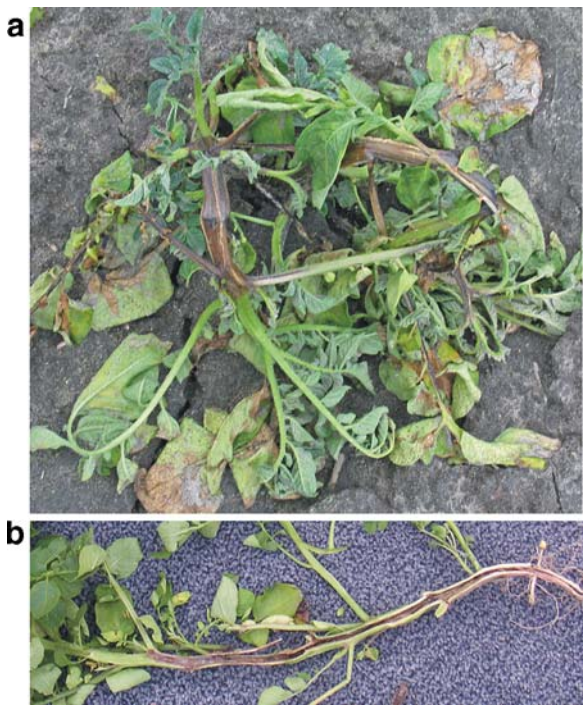

Fig. 6 Blackleg-like rotting (**a**) and internal necrosis of the stem (**b**) caused by *Dickeya* sp. strains D w0443 and D w04K, respectively

Table 2 Symptoms in field test with artificially inoculated tubers

Strains	Non-emergence (%)	Rotting or necrosis of the stem (%)
Group I		
D s0411	0	7
D s0413	7	25
D s0432-1	13	27
D s0433	14	29
D t042	0	7
D w0443	0	23
Group II		
D w04K	5	86
D w04M	5	60
Group III		
D w0431	0	4
D w0440	6	9
Pa s035-2	5	13
Water	0	3

Results are shown as percentage of the plants showing non-emergence and rotting of the stem until the end of growing season across seven replications.

16S sequences were determined from some of the Finnish strains to enable comparison with published 16S sequences. The 16S sequences and the growth at 39°C suggested that strains in group II were *D. dianthicola*, but the remaining species did not cluster well with the known *Dickeya* species. Blast search with 16S sequences in groups I and III in GenBank did not reveal identical sequences. The sequences that resembled group I sequences most were from strain 580, isolated from *Syngonium podophyllum* (AF373202, Fessehaie et al. 2002) and S3-1, isolated from *Zantedeschia aethiopica* (AY360397, Lee and Chen 2002), both ornamentals in the *Araceae*. The two strains originating from potato in the 16S tree, 340 and 571 (Fessehaie at al. 2002) clustered in *D. chrysanthemi* and *D. zeae*, respectively. It is possible that the strains in the Finnish group I have recently been adapted to potato or are characteristic of a cool climate and therefore they have not been characterised earlier. According to the homepage of CFBP, the *D. zeae* type strain was isolated in 1970 and the other *Dickeya* type strains at least ten years earlier, and several of them are from the tropics and the remainder from the USA.

Sequences of the group III strains were not identical with any previously published sequences in databases, and the strains contained a unique 11 bp insert in their 16S-23S sequence not found in the other *Dickeya* sequences. Bacteria in group III were not isolated from potato samples and were not particularly virulent on potato when compared to the other groups, and may therefore not originate from potato. Cother et al. (1992) also reported that not all *Dickeya* strains isolated from water were able to infect potato. The origin of the non-virulent water strains could be greenhouse cultivation of ornamental plants, or they may be part of the natural microflora (Cother et al. 1992).

The geographical distribution of the groups in water samples varied, and from each river strains from only one of the groups were isolated. *Dickeya dianthicola*-like strains were found in the rivers in the eastern part of the coastal areas not belonging to the major potato production district. These strains produced the highest incidence of blackleg-like symptoms in the field test. Two strains (*Dickeya* s053-3 and *Dickeya* s053-6) belonging to this group were isolated from potatoes in 2005, but they were sequenced and classified after the onset of field tests and therefore they were not included in the virulence tests. The finding of group II isolates in potato samples suggests that even isolates in this group originate from potato, or alternatively, may spread to potato. Strains classified in group I were isolated from potato samples collected from the major potato cultivation area, but also isolated from two water samples from southern Finland, whereas the strains in group III were found in many rivers in western parts of the country with commercial potato production but were never found from potatoes. There seemed to be no correlation between potato cultivation and the type and virulence of the *Dickeya* isolated from the rivers. The reason for this is unknown. However, our results suggest that different *Dickeya* strains exist in different rivers, as has been seen elsewhere (Cother et al. 1992), and support the theory that river water may contaminate clean potato stocks if used for irrigation (Cappaert et al. 1988).

This study demonstrates that *Dickeya* is a problem in potato production also in northern Europe and not only in warm countries in continental Europe. Furthermore, our results suggest that there is large genetic variation within the *Dickeya* genus and not all strains may have been included within the six *Dickeya* species characterised to date (Samson et al. 2005).

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References

Avrova, A. O., Hyman, L. J., Toth, R. L., & Toth, I. K. (2002). Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. *Applied & Environmental Microbiology*, *68*, 1499–1508.

Boccara, M., Vedel, R., Lalo, D., Lebrun, M. H., & Lafay, J. F. (1991). Genetic diversity and host range in strains of *Erwinia chrysanthemi*. *Molecular Plant Microbe Interactions*, *4*, 293–299.

Cappaert, M. R., Powelson, M. L., Franc, G. D., & Harrison, M. D. (1988). Irrigation water as a source of inoculum of soft rot erwinias for aerial stem rot of potatoes. *Phytopathology*, *78*, 1668–1672.

Cother, E. J., Bradley, J. K., Gillings, M. R., & Fahy, P. C. (1992). Characterization of *Erwinia chrysanthemi* biovars in alpine water sources by biochemical properties, GLC fatty acid analyses and genomic DNA fingerprinting. *Journal of Applied Bacteriology*, *73*, 99–107.

DeBoer, S. H., & Ward, L. J. (1995). PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology*, *85*, 854–858.

Dupuis, B., Michelante, D., Garcia, N., Nimal, C., & Stilman, D. (2005). Evolution of *Erwinia*'s potato contaminations in the Walloon Region during the seasons 2003 and 2004: Analysis of field and storage infections. Paper presented at the 16th triennial conference of the EAPR, European Association for Potato Research, Bilbao, Spain.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Fessehaie, A., De Boer, S. H., & Levesque, C. A. (2002). Molecular characterization of DNA encoding 16S-23S rRNA intergenic spacer regions and 16S rRNA of pectolytic *Erwinia* species. *Canadian Journal of Microbiology*, *48*, 387–398.

Gardan, L., Gouy, C., Christen, R., & Samson, R. (2003). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *53*, 381–391.

Hasegawa, M., Kishino, H., & Yano, T. (1985). Dating the human–ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, *22*, 160–174.

Hélias, V., Le Roux, A. C., Boishardy, M., Aujean, J. M., Copin, P., Perramant, M., et al. (2004). Erwinias causing blackleg in France: Species/subspecies and their distribution in 2003. Paper presented at the E.A.P.R. pathology section meeting, Lille, France.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*, 754–755.

Janse, J. D., & Ruissen, M. A. (1988). Characterization and classification of *Erwinia chrysanthemi* strains from several hosts in the Netherlands. *Phytopathology*, *78*, 800–808.

Lee, Y.-A., & Chen, K.-P. (2002). First report of bacterial soft rot of white flowered calla lily caused by *Erwinia chrysanthemi* in Taiwan. *Plant Disease*, *86*, 1273–1273.

Littell, R. C., Milliken, G. A., Stroup, W. W., & Wolfinger, R. D. (1996). *SAS system for mixed models*. Cary, NC: SAS Institute Inc.

Nassar, A., Darrasse, A., Lemattre, M., Kotoujansky, A., Dervin, C., Vedel, R., et al. (1996). Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of pel genes. *Applied and Environmental Microbiology*, *62*, 2228–2235.

Norman, D. J., Yuen, J. M. F., Resendiz, R., & Boswell, J. (2003). Characterization of *Erwinia* populations from nursery retention ponds and lakes infecting ornamental plants in Florida. *Plant Disease*, *87*, 193–196.

Palacio-Bielsa, A., Cambra, M. A., & Lopez, M. M. (2006). Characterisation of potato isolates of *Dickeya chrysanthemi* in Spain by a microtitre system for biovar determination. *Annals of Applied Biology*, *148*, 157–164.

Pérombelon, M. C. M. (2002). Potato diseases caused by soft rot erwinias: An overview of pathogenesis. *Plant Pathology*, *51*, 1–12.

Pérombelon, M. C. M., & Hyman, L. J. (1987). Frequency of *Erwinia carotovora* in the Alyth Burn in eastern Scotland and the sources of the bacterium. *Journal of Applied Bacteriology, 63*, 281–291.

Pérombelon, M. C. M., & van der Wolf, J. M. (Eds.) (2002). Methods for the detection and quantification of *Erwinia carotovora* subsp. atroseptica (*Pectobacterium carotovorum* subsp. *atrosepticum*) on potatoes: A laboratory manual. Invergowrie, Dundee, Scotland: Scottish Crop Research Institute.

Persson, P. (1991). Soft rot *Erwinia* species attacking potatoes in Sweden. Plant Protection Reports Dissertations 20. Swedish University of Agricultural Sciences.

Pirhonen, M., & Palva, E. T. (1988). Occurrence of bacteriophage T4 receptor in *Erwinia carotovora*. *Molecular and General Genetics*, *214*, 170–172.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*, 1572–1574.

Samson, R., Legendre, J. B., Christen, R., Fischer le Saux, M., Achouak, W., & Gardan, L. (2005). Transfer of *Pecto-*

bacterium chrysanthemi (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 1415–1427.

Schaad, N. W., Jones, J. B., & Chun, W. (Eds.) (2001). Laboratory guide for identification of plant pathogenic bacteria. St. Paul, Minnesota, USA: The American Phytopathological Society.

Toth, I. K., Avrova, A. O., & Hyman, L. J. (2001). Rapid identification and differentiation of the soft rot erwinias by 16S-23S intergenic transcribed spacer-PCR and restriction fragment length polymorphism analyses. *Applied & Environmental Microbiology*, *67*, 4070–4076.

Toth, I. K., Bell, K. S., Holeva, M. C., & Birch, P. R. J. (2003). Soft rot erwiniae: From genes to genomes. *Molecular Plant Pathology*, *4*, 17–30.

Waleron, M., Waleron, K., Podhajska, A. J., & Lojkowska, E. (2002). Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analysis of a recA gene fragment. *Microbiology*, *148*, 583–595.