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Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp. strains isolated from potato in Europe

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Abstract Sixty-five potato strains of the soft rot-causing plant pathogenic bacterium *Dickeya* spp., and two strains from hyacinth, were characterised using biochemical assays, REP-PCR genomic finger printing, 16S rDNA and *dnaX* sequence analysis. These methods were compared with nineteen strains representing six *Dickeya* species which included the type strains. A group of twenty-two potato strains isolated between 2005-2007 in the Netherlands, Poland, Finland and Israel were characterised as belonging to biovar 3. They were 100% identical in REP-PCR, *dnaX* and 16S rDNA sequence analysis. In a polyphasic analysis they formed a new clade different from the six *Dickeya* species previously described, and may therefore constitute a new species. The strains were very similar to a Dutch strain from hyacinth. On the basis of *dnaX* sequences and biochemical assays, all other potato strains isolated in Europe between 1979 and 1994 were identified as *D. dianthicola* (biovar 1 and 7), with the exception of two German strains classified as *D. dieffenbachia* (biovar 2) and *D. dadantii* (biovar 3), respectively. Potato strains from Peru were classified as *D. dadantii*, from Australia as *D. zeae* and from Taiwan as *D. chrysanthemi* bv. *parthenii*, indicating that different *Dickeya* species are found in association with potato.

Keywords Blackleg · *DnaX* · *Erwinia chrysanthemi* · *Pectobacterium chrysanthemi* · Soft rot

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Introduction

Dickeya spp. (Samson et al. 2005), formerly named *Pectobacterium chrysanthemi* (Hauben et al. 1998) and *Erwinia chrysanthemi* (Burkholder et al. 1953) are the causal agents of stem wet rot and blackleg in potato. These bacteria can also cause soft rot during transit and storage. The pathogen currently causes increasing economic damage in potato in different countries in Europe (Van der Wolf and De Boer 2007). The genus *Dickeya* is a diverse group, which can affect a high number of different plant species, including many economically important crops. Strains from different host plants can differ in host range and in pathogenic and phenotypic properties (Dickey 1979, 1981; Samson et al. 2005). Strains from the same host plant also can belong to different biochemically distinctive groups (biovars) and species.

Isolates of the pathogen have been recently reclassified into six species within the genus *Dickeya* on the basis of different features including homology in DNA-DNA hybridisation analysis (Samson et al. 2005). This classification is partly correlated with the former distribution in biochemically distinct groups (biovars) described by Ngwira and Samson (1990). Studies in the past indicated that European potato strains often belonged to biovars 1 and 7, which coincide with *D. dianthicola*, a species adapted to cool regions (Janse and Ruissen 1988). Potato strains from other continents belonged to biovars 3 and 6, and possessed a higher optimum growth temperature (Dickey 1981; Hsu and Tzeng 1981; Cother and Powell 1983).

Recently, additional *Dickeya* variants have been found in potatoes from Europe. Laurila et al. (2008) described the presence of a *Dickeya* group, clearly divergent from *D. dianthicola* on the basis of 16S-23S rDNA internal transcribed spacer (ITS) sequences. Strains belonging to this new clade were on average more virulent in greenhouse and potato tuber slice assays than *D. dianthicola*, although variability was high. In contrast, strains from this group were less virulent in a field experiment in Finland with vacuum-infiltrated tubers. Also in Spain strains divergent from *D. dianthicola*, belonging to biovars 3 and 6, were isolated from potato (Palacio-Bielsa et al. 2006). Tsror et al. (2008) described the isolation of biovar 3 strains from blackleg-diseased potato plants in Israel, grown from Dutch seed. These strains were similar in rep-PCR analysis and biochemical assays as strains recently isolated from seed potatoes in the Netherlands. Sławiak et al. (2009) reported for the first time the presence of *Dickeya* sp. biovar 3 strains from blackleg-diseased potato plants in Poland.

This research evaluates the diversity of strains of *Dickeya* spp. isolated from potato in Europe in a period from 1979 to 2007. We developed a method for rapid characterisation of *Dickeya* species based on *dnaX* sequence analysis. We used this technique in addition to 16S rDNA sequence analysis, REP-PCR and biovar determination to characterise 65 *Dickeya* spp. from potato. A set of strains, representing the six named *Dickeya* species including the type strains, were used as a reference. We found a homogeneous new cluster of potato strains, which did not fit in any of the described six *Dickeya* species. The implications of our findings with respect to dissemination, plant breeding and risk assessment are discussed.

Materials and methods

Bacterial strains

Nineteen strains representing *D. chrysanthemi, D. paradisiaca, D. dadantii, D. dianthicola, D. dieffenbachia*, and *D. zeae* (Samson et al. 2005), including the designated type strain for each species and 67 *Dickeya* strains from the IPO collection (Plant Research International) were used in this study (Table 1). Most of the IPO strains (43) were isolated in Europe from potato between 1979 and 2007.

Confirmation of the identity

The identity of the strains as belonging to *Dickeya* spp. (previous *Erwinia chrysanthemi*) was evaluated by testing the pectinolytic activity, growth at 37°C, production of phosphatases, ability of acid production from α-methyl-glucoside and by PCR with *pelADE* specific primers (Nassar et al. 1996).

Biochemical assays

The bacterial strains were classified using the microtiter plate assay described by Palacio-Bielsa et al. (2006) with few modifications as described below. It included growth at 39°C, 41°C and 25°C (control) on nutrient broth (NB, Oxoid) (Dye 1968), anaerobic hydrolysis of arginine (Moeller 1955), and polysaccharide inulin utilisation in phenol red peptone water (inulin extracts from chicory and dahlia were used at 0.3% final concentration). Eight carbon sources were tested by acidification/alkalisation on liquid Ayers, Rupp and Johnson medium (Ayers et al. 1919) with bromothymol blue mixed with different 0.3% carbohydrates: (−)-D-arabinose, 5-keto-D-gluconate, mannitol, (+)-D-melibiose, (+)-D-raffinose and (−)-D-tartrate, β-gentiobiose and (+)-L-tartrate. The different basal media (150 µl) were dispensed on a sterile culture microplate (Greiner bio-one, Cellstar), and 15 µl of bacteria suspension of 10^8 cells ml^{-1} of each isolate to be analysed were added per well. Wells which contained arginine, were covered with a layer (100 µl per well) of sterile glycerol to obtain anaerobic conditions. All 96 well microplates were wrapped in parafilm. Plates were incubated at 25°C for 72 h, except for (−)-D-arabinose, for which an incubation period of 96 h was used. Plates were

Table 1 Strains of the *Dickeya* spp. (ex *Erwinia chrysanthemi* and *Pectobacterium chrysanthemi*) used in this study

IPO no.	CFBP no.	Host plant, cultivar	Geographical origin, location, year of isolation, other collection numbers	Biovar	*Dickeya* spp.	Predicted genomic species on the basis of dnaX sequence analysis
Reference strains (Samson et al. 2005)						
2114	**1200^T**	***Dianthus caryophyllus***	**UK, 1956**	**1**	**5 *(D. dianthicola)***	***D. dianthicola***
2115	1276	*Dahlia* sp.	Romania, 1962	a	a *(D. dianthicola)*	*D. dianthicola*
2116	2015	*Solanum tuberosum*	France, 1975	7	5 *(D. dianthicola)*	*D. dianthicola*
2117	1236	*Parthenium argentatum*	USA, 1945	6	3 *(D. chrysanthemi* bv. *parthenii)*	*D. chrysanthemi* bv. *parthenii*
2118	**2048^T**	***Chrysanthemum morifolium***	**USA, 1958**	**5**	**3 *(D. chrysanthemi* bv. *chrysanthemi)***	***D. chrysanthemi* bv. *chrysanthemi***
2119	3703	*Helianthus annuus*	France, 1986	5	3 *(D. chrysanthemi* bv. *chrysanthemi)*	*D. chrysanthemi* bv. *chrysanthemi*
2120	**1269^T**	***Pelargonium capitatum***	**Comoros, 1960**	**3**	**2 *(D. dadantii)***	***D. dadantii***
2121	1278	*Ananas comosus*	Malaysia, 1961	3	a *(D. dadantii)*	*D. dadantii*
2122	3697	*Ipomoea batatas*	Cuba, 1987	a	a *(D. dadantii)*	*D. dadantii*
2124	1360	*Dieffenbachia* sp.	France, 1972	2	4 *(D. dieffenbachiae)*	*D. dieffenbachiae*
2125	**2051^T**	***Dieffenbachia* sp.**	**USA, 1957**	**2**	**4 *(D. dieffenbachiae)***	***D. dieffenbachiae***
2126	3694	*Lycopersicon esculentum*	Cuba, 1987	2	4 *(D. dieffenbachiae)*	*D. dieffenbachiae*
2127	**3477^T**	***Musa paradisiaca***	**Colombia, 1968**	**4**	**6 *(D. paradisiaca)***	***D. paradisiaca***
2128	3699	*Zea mays*	Cuba, 1987	4	6 *(D. paradisiaca)*	*D. paradisiaca*
2129	4178	*Musa paradisiaca*	Colombia, 1970	a	a *(D. paradisiaca)*	*D. paradisiaca*
2130	1537	*Saccharum officinarum*	Australia	3	a *(Dickeya* spp.*)*	*Dickeya* spp.
2131	**2052^T**	***Zea mays***	**USA, 1970**	**3**	**1 *(D. zeae)***	***D. zeae***
2132	4176	*Chrysanthemum morifolium*	USA, 1970	3	1 *(D. zeae)*	*D. zeae*
2133	6466	*Ananas comosus*	Martinique (Fr.), 1991	3	1 *(D. zeae)*	*D. zeae*
Dickeya spp. strains used in this study						
502		*Solanum tuberosum* Astarte	The Netherlands, 1979 226[a]	7		*D. dianthicola*
597		*Solanum tuberosum*	Peru, 1979 30[b]	3		*D. dadantii*
598		*Solanum tuberosum*	Peru, 1979 32[b]	3		*D. dadantii*
648		*Solanum tuberosum*	Australia, 1980 30513[c]	3		ns
649		*Solanum tuberosum*	Australia, 1980 30514[c]	3		*D. zeae*
650		*Solanum tuberosum*	Australia, 1980	3		*D. zeae*

Table 1 (continued)

IPO no.	CFBP no.	Host plant, cultivar	Geographical origin, location, year of isolation, other collection numbers	Biovar	*Dickeya* spp.	Predicted genomic species on the basis of dnaX sequence analysis
			30515[c]			
651		*Solanum tuberosum*	Australia, 1980 78/14[c]	3		*D. zeae*
655		*Solanum tuberosum*	Taiwan, 1980 1591 SP1[d]	6		*D. chrysanthemi* bv. *parthenii*
713		*Solanum tuberosum* Elkana	The Netherlands, 1983	7		*D. dianthicola*
754		*Solanum tuberosum*	Peru, 1983 4[b]	3		*D. dadantii*
775		*Solanum tuberosum* Saturna	The Netherlands, 1984 924[a]	7		*D. dianthicola*
838		*Solanum tuberosum* Bintje	The Netherlands, 1987 484[a], 303[e]	1		*D. dianthicola*
839		*Solanum tuberosum*	The Netherlands, 1987 486[a], 573[e]	7		*D. dianthicola*
846		*Solanum tuberosum* Ostara	The Netherlands, 1987 483[a], 181[e], 3542[f]	7		*D. dianthicola*
871		*Solanum tuberosum*	The Netherlands, 1988 483[a]	7		*D. dianthicola*
872		*Solanum tuberosum*	The Netherlands, 1988 484[a]	1		*D. dianthicola*
878		*Solanum tuberosum*	The Netherlands, 1988 499[a]	1		*D. dianthicola*
879		*Solanum tuberosum*	The Netherlands, 1988 581[a]	7		*D. dianthicola*
912		*Solanum tuberosum*	France, Bretagne, 1988 85.6.21.c[g]	1		*D. dianthicola*
913		*Solanum tuberosum*	France, Bretagne, 1988 85.5[g]	7		*D. dianthicola*
976		*Solanum tuberosum* Marfona	The Netherlands, Tollebeek, 1991	7		*D. dianthicola*
978		*Solanum tuberosum* Elkana	The Netherlands, 1991	1		*D. dianthicola*
980		*Solanum tuberosum* Arkula	The Netherlands, Tollebeek, 1991	7		*D. dianthicola*

981	*Solanum tuberosum* Elkana	The Netherlands, 1991	7	*D. dianthicola*
982	*Solanum tuberosum* Irene	The Netherlands, Tollebeek, 1991	1	*D. dianthicola*
992	*Solanum tuberosum* Ostara	The Netherlands, 1991	7	*D. dianthicola*
998	*Solanum tuberosum* Morene	The Netherlands, 1986 751[a]	a	*P. atrosepticum*
1108	*Solanum tuberosum* Sante	The Netherlands, 1994 3710[f]	7	*D. dianthicola*
1109	*Solanum tuberosum* Sante	UK, 1994 3881[f]	7	*D. dianthicola*
1259	*Solanum tuberosum*	Germany, Ungarn DF 5[h]	2	*D. dieffenbachiae*
1260	*Solanum tuberosum*	Germany, Braunschweig 179[h]	3	*D. dadantii*
1301	*Solanum tuberosum*	Spain, Valencia 1360-19[i]	1	*D. dianthicola*
1302	*Solanum tuberosum*	Spain, Valencia 1360-20[i]	1	*D. dianthicola*
1348	*Solanum tuberosum*	The Netherlands, 1993	1	*D. dianthicola*
1350	*Solanum tuberosum*	The Netherlands, 1994	1	*D. dianthicola*
1351	*Solanum tuberosum*	The Netherlands, 1994	1	*D. dianthicola*
1478	*Solanum tuberosum* Edzina	Spain, Almacera 1360-2236[i]	1	*D. dianthicola*
1493	*Solanum tuberosum*	Spain, Meliana 1374-62a[i]	1	*D. dianthicola*
1739	*Solanum tuberosum*	The Netherlands, 1992	7	*D. dianthicola*
1741	*Solanum tuberosum*	The Netherlands, 1992	1	*D. dianthicola*
2017	*Hyacinthus* sp.	The Netherlands I31[j]	3	*D. dadantii*
2019	*Hyacinthus* sp.	The Netherlands H4[j]	3	new clade
2093	*Solanum tuberosum*	Finland, 2005 04V043[k], w0443[k]	3	new clade
2094	*Solanum tuberosum*	Finland, 2005 04V061[k], w0461[k]	3	new clade
2095	*Solanum tuberosum*	Finland, Loviisa, 2005 04Loviisa1[k], w04L[k]	1	ns

Table 1 (continued)

IPO no.	CFBP no.	Host plant, cultivar	Geographical origin, location, year of isolation, other collection numbers	Biovar	*Dickeya* spp.	Predicted genomic species on the basis of dnaX sequence analysis
2096		*Solanum tuberosum*	Finland, Mustijoki, 2005 04Mustijoki3[k], w04M[k]	1		*D. dianthicola*
2097		*Solanum tuberosum*	Finland, Koskenkyl, 2005 04Koskenkyl2[k], w04K[k]	7		*D. dianthicola*
2098		*Solanum tuberosum*	Finland, 2005 05003-2a[k], s053-2[k]	3		new clade
2187		*Solanum tuberosum* Sapphire	Israel, Nir Itshak, 2006 G-87[l]	3		new clade
2188		*Solanum tuberosum* Desiree	Israel, Nevatim, 2006 G-118[l]	3		new clade
2189		*Solanum tuberosum* Sante	Israel, Kisufim, 2006 G-115[l]	3		new clade
2190		*Solanum tuberosum* Sante	Israel, Kisufim, 2006 G-122[l]	3		new clade
2191		*Solanum tuberosum* Quinsy	Israel, Or Haner, 2006 G-120[l]	3		new clade
2192		*Solanum tuberosum* Quinsy	Israel, Kerem Shalom, 2006 G-121[l]	3		new clade
2222		*Solanum tuberosum* Melody	The Netherlands, 2007 202	3		new clade
2225		*Solanum tuberosum* Kondor	The Netherlands, 2007 256	3		new clade
2234		*Solanum tuberosum*	Israel, 2007 P.C 3[l]	3		new clade
2235		*Solanum tuberosum*	Israel, 2007 P.C 26.4[l]	3		new clade
2236		*Solanum tuberosum*	Israel, 2007 P.C 36[l]	3		new clade
2237		*Solanum tuberosum*	Israel, 2007 P.C 41.4[l]	3		new clade
2238		*Solanum tuberosum*	Israel, 2007 Imp. 1[l]	3		new clade
2239		*Solanum tuberosum*	Israel, 2007 Imp. 23.1[l]	3		new clade
2240		*Solanum tuberosum*	Israel, 2007	3		new clade

		Imp. 24[l]		
2241	*Solanum tuberosum*	Israel, 2007 G169[l]	3	new clade
2276	*Solanum tuberosum* Sante	Poland, Podlaskie, 2005 101A/9/2005[m]	3	new clade
2277	*Solanum tuberosum* Sante	Poland, Podlaskie, 2005 101A/11/2005[m]	3	new clade
2278	*Solanum tuberosum* Sante	Poland, Podlaskie, 2005 101A/12/2005[m]	3	new clade

a Ambiguous results.; ns not sequenced.; nt not tested.; Type strains are marked in bold

[a] PD, Collection of Plant Protection Service, Wageningen, The Netherlands

[b] CIP, International Collection, Centro Internacional de la Papa, Lima, Peru

[c] DAR, Australian Collection of Plant Pathogenic Bacteria, Australia

[d] Dickey Cornell University, Ithaka, USA

[e] NAK, Dutch General Inspection Service for Agricultural Seeds and Seed Potatoes, Emmeloord, The Netherlands

[f] NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK

[g] INRA, Institut National de la Recherche Agronomique, Angers, France

[h] BBA, Federal Research Centre for Agriculture and Forestry, Braunschweig, Germany

[i] IVIA, Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain

[j] PPO, Applied Plant Research, Wageningen, The Netherlands

[k] MTT, Agrifood Research Finland, Joioinen, Finland

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observed every 24 h and tests were completed after 120 h of incubation. Tests were performed three times and each time four wells per assay were used. Strains IPO 981 (biovar 7), IPO 982 (biovar 1) and IPO 2017 (biovar 3) were used as controls in each microplate.

Development of a *dnaX* primer set

Specific PCR primers for the amplification of *dnaX* were developed by comparing the *dnaX* sequence of *Pectobacterium atrosepticum,* SCRI 1043 (gene 1330287-1332362, BX950851) with sequences in the nucleotide database of the National Centre for Biotechnology Information (NCBI). This comparison was performed by using the Basic Local Alignment Search Tool (BLAST). A degenerated forward (dnaXf forward, 5'-TATCAGGTYCTTGCCCGTAAGTGG-3') and reverse primer (dnaXr, 5'-TCGACATCCARCG CYTTGAGATG-3') were designed from the most preserved regions. PCR amplification using dnaXf and dnaXr resulted in a product with a length of 535 bp.

DNA extraction and PCR amplification

Bacterial genomic DNA was isolated from bacterial suspensions in sterile distilled water (10^8 cells ml^{-1}), prepared from 48-h cultures on TSA medium (Tryptone Soya Agar, Oxoid). The DNA was purified using the silica beads method (Bertheau et al. 1998). For 16S rDNA fragment gene amplification PCR primers F985PTO (5'-AACGCGAAGAACCTTAC-3') and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') were used (Heuer et al. 1999). PCR was performed in 50 µl of a reaction mixture containing 1 × PCR buffer, 200 µM dNTPs, 0.4 µM of each primer, 1 U Taq DNA polymerase (Roche Diagnostics GmbH) and 2 µl of template DNA. The amplification programme for primers F985PTO and R1378 consisted of an initial denaturation (94°C, 3 min) followed by 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min) with a single final extension step (72°C, 5 min). PCR amplification for *dnaX* genes was similar, but the annealing was performed at 59°C for 1 min. PCR products were analysed on 2% agarose gel containing with ethidium bromide.

Phylogenetic analyses

The PCR products were purified on clean-up columns (Qiagen) according to the manufacturer's instructions. After purification, amplicons were sequenced from the forward and reverse primers. A consensus sequence for each strain was created. *DnaX* gene sequences of 65 strains were compared with sequences from 19 reference strains. 16S rDNA gene sequences of 64 strains were compared with *Dickeya*

Table 2 Reaction patterns of 19 reference *Dickeya* spp. strains and 67 *Dickeya* spp. strains from the culture collection of Plant Research International, Wageningen, The Netherlands (see Table 1) in a microplate assay for biovar determination

Biovar	1	2	3	3	3	4	5	6	7
Genomic species	5	4	1	2	unknown	6	3	3	5
Test									
(−)-D-Arabinose	−	V (57)	+	+	+	+	−	−	−
(−)-D-Tartrate	+	−	−	−	−	−	−	−	V (89)
Inulin (from chicory)	−	−	−	−	−	−	+	−	−
Inulin (from dahlia)	−	−	−	−	−	−	+	−	−
(+)-D-Melibiose (+)-D-Raffinose	+	−	+	+	+	+	+	+	−
5-Keto-D-gluconate	−	−	−	−	−	+	−	−	−
Mannitol	+	+	+	+	+	−	+	+	+
β-Gentiobiose	−	+	−	V (17)	+w	+	−	−	−
(+)-L-Tartrate	−	−	V (46)	−	−	−	−	−	−
Arginine dihydrolase	+	−	−	−	−	−	V (80)	−	V (89)
Growth at 39°C on NB	−	+	+	+	+w	+	+	+	−
Growth at 41°C on NB	−	−	+	−	−	−	+	+	−
Growth at 25°C on NB (control)	+	+	+	+	+	+	+	+	+

+ 100% of the strains positive.; − 100% of the strains negative.; V (n) Variable (*n* = percentage of positive strains).; +w Weak growth or weak positive reaction

spp. sequences available in the public data base (http://www.ncbi.nlm.nih.gov/). Additionally, *P. atrosepticum* 16S rDNA gene sequences from SCRI 1043 and IPO 161 strains (BX950851 and AY914794) and *P. atrosepticum dnaX* gene sequence from strain SCRI 1043 (BX950851) for 16S rDNA and *dnaX* sequences analysis were included. All sequences for the 16S rDNA (421 nucleotides) and *dnaX* (535 nucleotides)

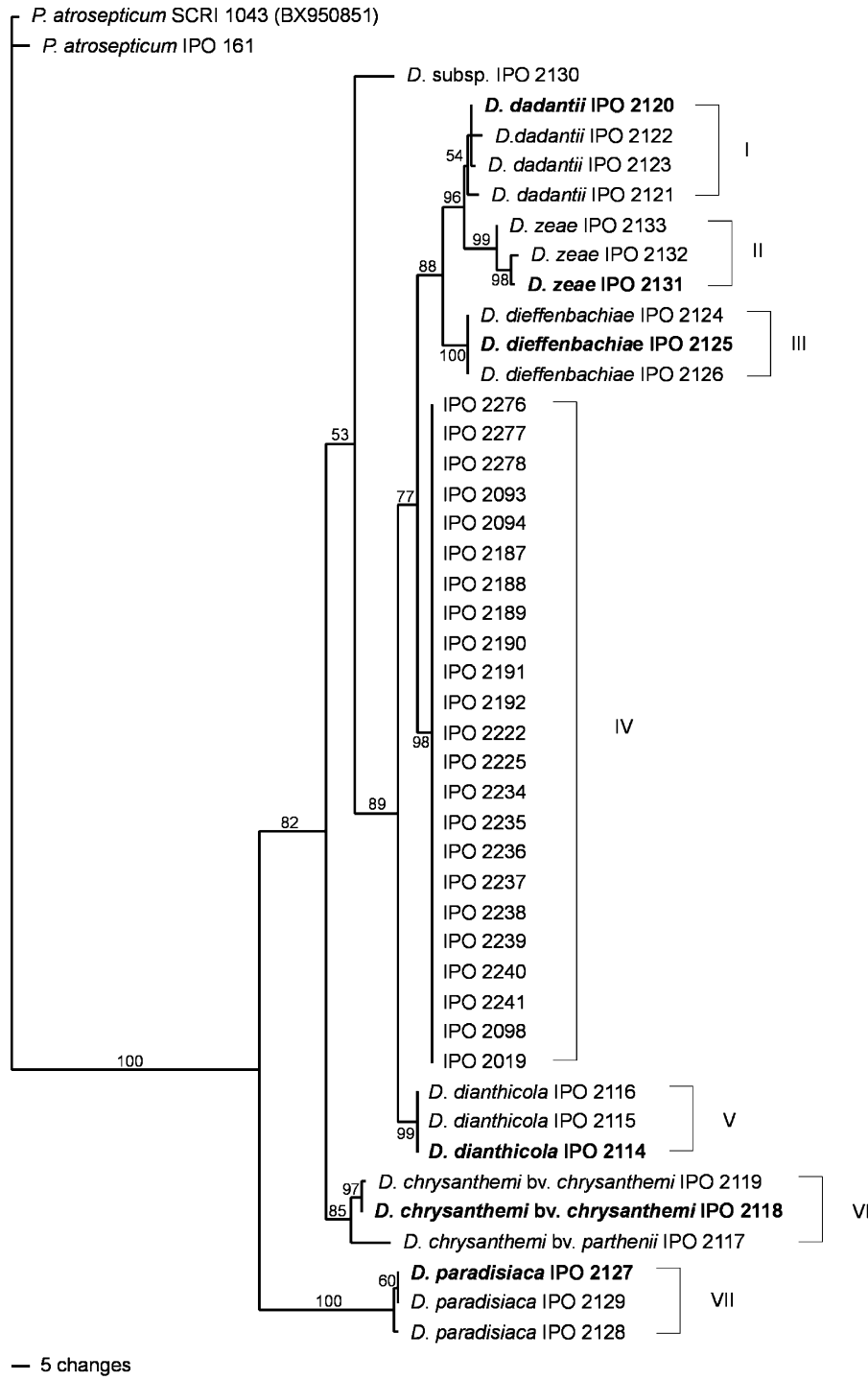
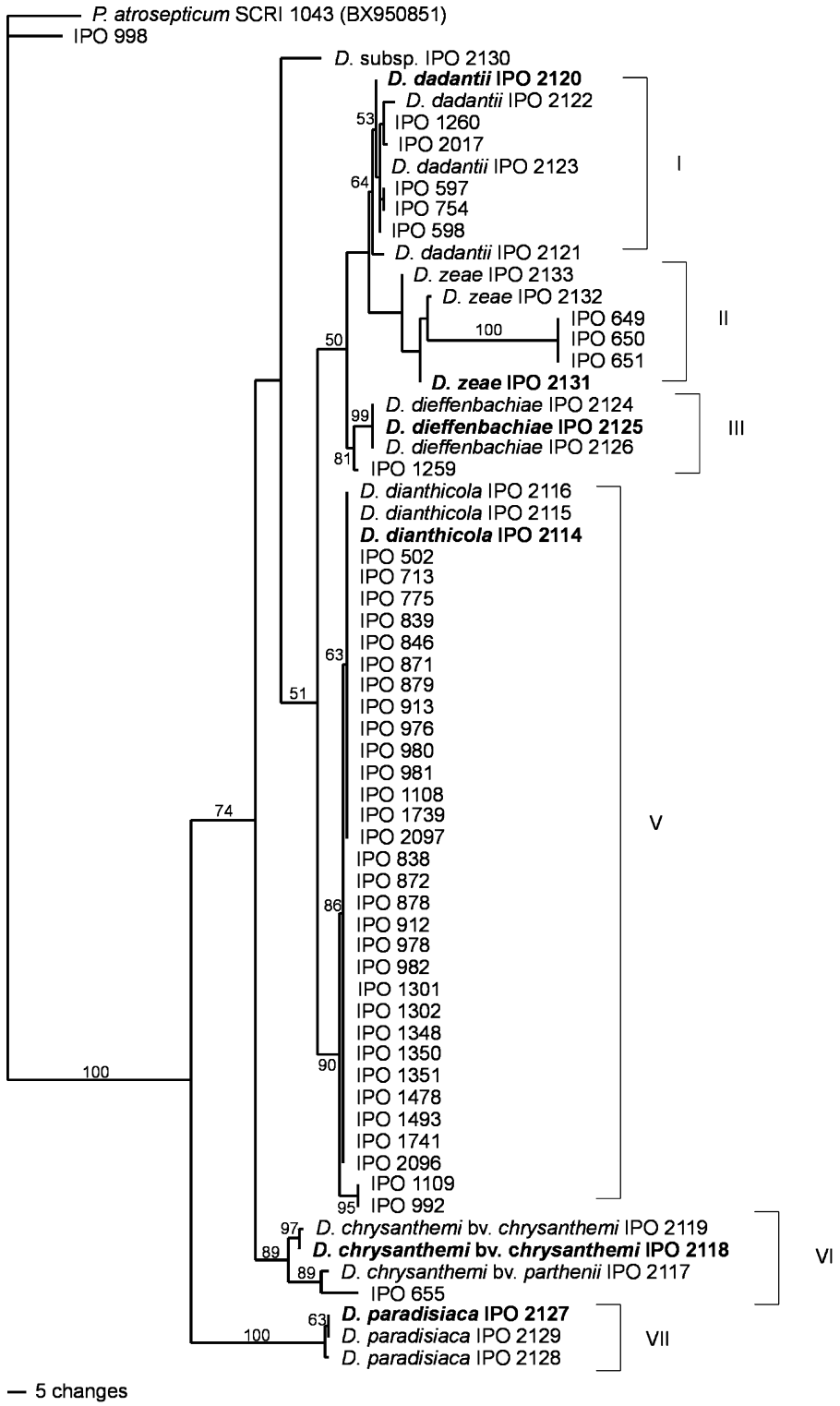


Fig. 1 Maximum parsimony phylogenetic analysis of *Dickeya* spp. strains based on *dnaX* gene sequences. Strains included type strains (in bold) and other reference strains representing the six *Dickeya* genomospecies. They also included biovar 3 potato strains recently isolated in Europe (clade IV) and a Dutch hyacinth strain (2019). *Pectobacterium atrosepticum* strains were used as out group. Branch lengths are proportional to the number of changes on a given branch, and bootstrap values are given for each node above 50%. Clades are indicated in Roman numerals


P. atrosepticum SCRI 1043 (BX950851)
IPO 998
D. subsp. IPO 2130
D. dadantii IPO 2120
D. dadantii IPO 2122
IPO 1260
IPO 2017
D. dadantii IPO 2123
IPO 597
IPO 754
IPO 598
D. dadantii IPO 2121
I
D. zeae IPO 2133
D. zeae IPO 2132
IPO 649
IPO 650
IPO 651
D. zeae IPO 2131
II
D. dieffenbachiae IPO 2124
D. dieffenbachiae IPO 2125
D. dieffenbachiae IPO 2126
IPO 1259
III
D. dianthicola IPO 2116
D. dianthicola IPO 2115
D. dianthicola IPO 2114
IPO 502
IPO 713
IPO 775
IPO 839
IPO 846
IPO 871
IPO 879
IPO 913
IPO 976
IPO 980
IPO 981
IPO 1108
IPO 1739
IPO 2097
IPO 838
IPO 872
IPO 878
IPO 912
IPO 978
IPO 982
IPO 1301
IPO 1302
IPO 1348
IPO 1350
IPO 1351
IPO 1478
IPO 1493
IPO 1741
IPO 2096
IPO 1109
IPO 992
V
D. chrysanthemi bv. chrysanthemi IPO 2119
D. chrysanthemi bv. chrysanthemi IPO 2118
D. chrysanthemi bv. parthenii IPO 2117
IPO 655
VI
D. paradisiaca IPO 2127
D. paradisiaca IPO 2129
D. paradisiaca IPO 2128
VII
53
64
50
100
99
81
51
63
74
86
90
100
97
89
89
63
100
95
5 changes

◀ **Fig. 2** Maximum parsimony phylogenetic analysis of *Dickeya* spp. strains based on *dnaX* gene sequences. Strains included in this analysis are the *Dickeya* type strains (in bold) and other reference strains representing the six *Dickeya* genomospecies. They also included all potato strains characterised in this study except the biovar 3 strains which are shown in Fig. 1. *Pectobacterium atrosepticum* strains were used as out group. Branch lengths are proportional to the number of changes on a given branch, and bootstrap values are given for each node above 50%. Clades are indicated in Roman numerals

genes were edited and aligned using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Successive phylogenetic trees were constructed to determine the relationship between strains. Four different methods (maximum parsimony, likelihood, distance and neighbour joining / UPGMA) were used for analysis. Final phylogenetic analysis for the 16S rDNA and *dnaX* data sets was carried out using maximum parsimony methods with PAUP (http://paup.csit.fsu.edu/about.html). For maximum parsimony methods, 1,000 bootstrap replicates were included in a heuristic search, with a random tree and the tree bisection-reconnection branch-swapping algorithm. The percent variation was calculated by comparing all isolates to the nearest relative.

REP-PCR genomic fingerprinting

Bacterial genomic DNA was isolated and purified as described for PCR amplification of 16S rDNA and *dnaX* fragment genes. REP-PCR conditions were used as described by Rademaker et al. (1998), with few modifications as described below. Primers REP1R (5'-IIIICGICGICATCIGGC-3') and REP2I (5'-ICGICTTATCIGGCCTAC-3') were used (Versalovic et al. 1991). The PCR mixture (27.075 µl) consisted of: 1 x PCR buffer (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 10.05 mM $MgCl_2$, 6.7 µM EDTA and 30 mM β-mercapto-ethanol), 1.13 mM dNTPs, 3.69 µM of each primer, 3 U Taq DNA polymerase (Roche Diagnostics GmbH) and 10 µl of template DNA. PCR amplifications were performed in a DNA Engine Peltier thermal cycler (Bio-Rad) with an initial denaturation (95°C, 7 min) followed by 40 cycles of denaturation (94°C, 1 min), annealing (40°C, 2 min), and extension (65°C, 8 min) with a single final extension step (65°C, 16 min). The PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Patterns were analysed using the Quantity One programme (Bio-Rad). A phenogram was constructed using the UPGMA (unweighted pair-group method using arithmetic means) tree building method.

Results

Validation of biochemical assays

Biovar determination of *Dickeya* spp. strains was done using the microtiter plate system described by Palacio-Bielsa et al. (2006). Additionally growth at 41°C was tested and the utilisation of two more carbon sources namely β-gentiobiose and (+)-L-tartrate (Samson et al. 2005) (Table 2). For 16 out of the 19 reference strains, results largely confirmed the biovar determination of Samson et al. (2005). For biovar 1 and 7 however, no inulin assimilation and for biovar 4 no acid production from (−)-D-tartrate was observed. Three strains gave ambiguous results (Table 1). Strain IPO 2115 (CFBP 1276) classified by Samson et al. (2005) as biovar 7 was similar to biovar 4 except for utilisation of (−)-D-arabinose. Strain IPO 2122 (CFBP 3697), classified as biovar 3, was similar to biovar 2 apart from utilisation of β-gentiobiose; Strain IPO 2129 (CFBP 4178) classified as biovar 4 was identical to biovar 3 (*D. zeae*).

Biochemical identification of strains

The validated biochemical microtiter assay was used for biovar determination of 67 selected *Dickeya* strains (Table 1). Most potato strains from Europe, including Israel, were classified into biovar 1, 3 and 7. One strain from a German potato field was typed as biovar 2. Potato strains from Peru and Australia were classified into biovar 3 and from Taiwan into biovar 6. Biovar 3 strains from potato recently isolated in Israel, Finland, Poland and the Netherlands were identical. They were also identical to a Dutch strain from hyacinth (IPO 2019) (Table 1), albeit different from other strains of ornamental plants including hyacinth (results not shown). Moreover, the new strains were also identical to other biovar 3 strains (*D. dadantii* and *D. zeae*) with the exception that they grew weakly at 39°C (Table 2).

Phylogenetic analysis of *dnaX* and 16S rDNA sequences

Phylogenetic analysis of 84 *Dickeya* strains including the 19 reference strains based on *dnaX* sequences revealed the presence of seven main groups (Fig. 1). The *DnaX* sequences of the biovar 3 potato strains from Israel, Finland, Poland and the Netherlands were identical and formed a clade (clade IV) distinct from the biovar 3 reference strains belonging to *D. dadantii* (clade I) and *D. zeae* (clade II) (Fig. 1). They were most similar to *D. dieffenbachia* (clade III) and the homology was 97%. The Dutch hyacinth strain IPO 2019 was identical to the biovars potato strains (clade IV) (Fig. 1). All strains typed as biovar 1 and 7 grouped tightly together with the *D. dianthicola* reference strains (clade V) and were distinct from other *Dickeya* species (Fig. 2). The biovar 2 potato strain from Germany (IPO 1259) grouped with the *D. dieffenbachiae* reference strains (clade III). Biovar 3 potato strains from Germany (IPO1260) and Peru (IPO597, IPO 598 and IPO 754) grouped with the *D. dadantii* reference strains (clade I) and from Australia (IPO 649, IPO 650 and IPO 651) grouped with the *D. zeae* reference strains (clade II). Biovar 6 potato strain (IPO 655) from Taiwan grouped with *D. chrysanthemi* pv. *parthenii* reference strain (clade VI) (Fig. 2). Moreover, based on *dnaX* sequence analysis biovar 3 hyacinth strain IPO 2017 grouped together with potato *D. dadantii* strains IPO 597, IPO 598, IPO 754 and IPO 1260 (clade I).

Cluster analysis of the 19 reference strains on basis of 16S rDNA did not show a clear relation with the species (Fig. 3). Again 16S rDNA sequences of the biovar 3 strains from Europe and Israel and one Dutch hyacinth strain IPO 2019 were identical and formed a cluster separately from other *Dickeya* species (Fig. 3).

REP-PCR genomic fingerprinting analysis

REP-PCR analysis followed by cluster analysis was done on a selection of eight biovar 3 strains recently isolated from potato: two from Israel (IPO 2187 and IPO 2234), two from Finland (IPO 2093 and IPO 2098), two from Poland (IPO 2276 and IPO 2277) and two from the Netherlands (IPO 2222 and IPO 2225). In the REP-PCR phenogram these eight strains formed a homogeneous clade distinct from other clades (Fig. 4). The Dutch biovar 3 strain IPO 2019 from hyacinth could also be separated from the biovar 3 potato strains. Biovar 3 strains isolated from potato in Peru and Germany (IPO 598, IPO 754 and IPO 1260) and one Dutch biovar 3 strain from hyacinth (IPO 2017) grouped with the *D. dadantii* reference strains; two biovar 3 strains from potato in Australia (IPO 649 and IPO 650) grouped with the *D. zeae* reference strains, and two biovar 1 and 7 strains from potato in the Netherlands (IPO 502 and IPO 982) grouped with the *D. dianthicola* reference strains.

Discussion

Sixty-five potato strains of *Dickeya* were analysed with biochemical and genetic techniques, including *dnaX* and 16S rDNA sequence analysis and REP-PCR genomic fingerprinting. A novel group of twenty-two strains within biovar 3 isolated between 2005 and 2007 was found, isolated from blackleg-diseased potato plants grown in Finland, Poland, the Netherlands and Israel. Strains were identical in all characters evaluated, formed a homogenous cluster after phylogenetic analysis and were similar to a strain isolated from hyacinth in 2002.

The *dnaX* analysis of the potato strains belonging to the new clade within biovar 3 grouped together with a Dutch biovar 3 strain from hyacinth (IPO 2019); they showed a 100% sequence identity. They were separated from the other six *Dickeya* species after cluster analysis of *dnaX* sequences and could not be identified to species level. They were most related to *D. dieffenbachiae* and clearly distinct from *D. zeae* and *D. dadantii* that comprise biovar 3 strains. The *dnaX* sequence analysis was chosen because it is the highest scoring candidate gene for predicting genome relatedness at the genus or subgenus level, among genes found in every bacterial genome so far sequenced (Zeigler 2003). The *dnaX* produces two subunits of DNA polymerase III and contains variable parts, flanked by highly conserved regions which are involved in a ribosomal frameshift (Blinkova et al. 1997). Phylogenetic analysis based on *dnaX* sequences confirmed the usefulness of *dnaX* in *Dickeya* taxonomy. The 19 reference strains, representing the 6 genomic *Dickeya* species grouped on DNA-DNA

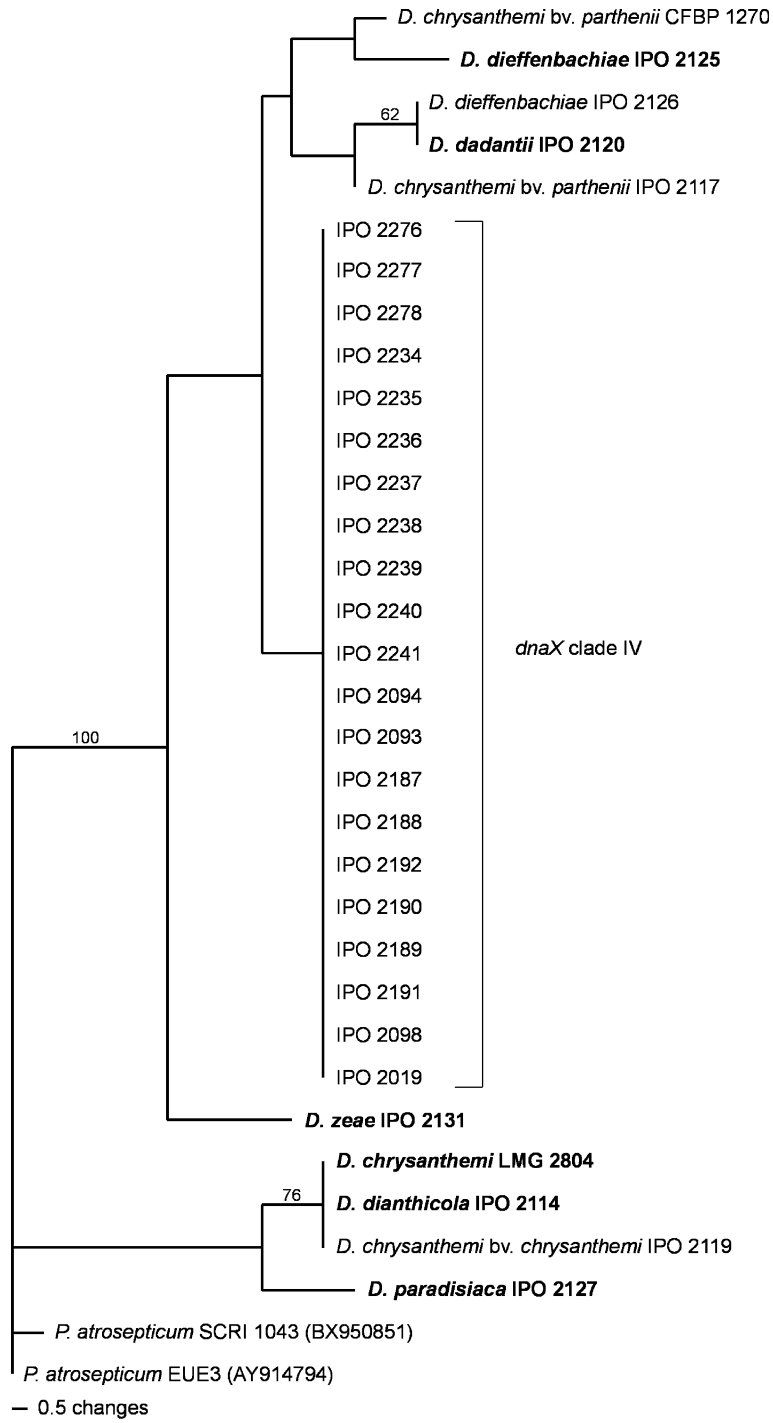


Fig. 3 Maximum parsimony phylogenetic analysis of the *Dickeya* spp. strains based on 16S rDNA gene sequences. In the analysis the *Dickeya*-type strains (in bold) and other reference strains representing the six *Dickeya* genomospecies are included. They also included biovar 3 potato strains recently isolated in Europe (clade IV) and a Dutch hyacinth strain (2019). Two *P. atrosepticum* strains were used as out group. Branch lengths are proportional to the number of changes on a given branch, and bootstrap values are given for each node above 50%

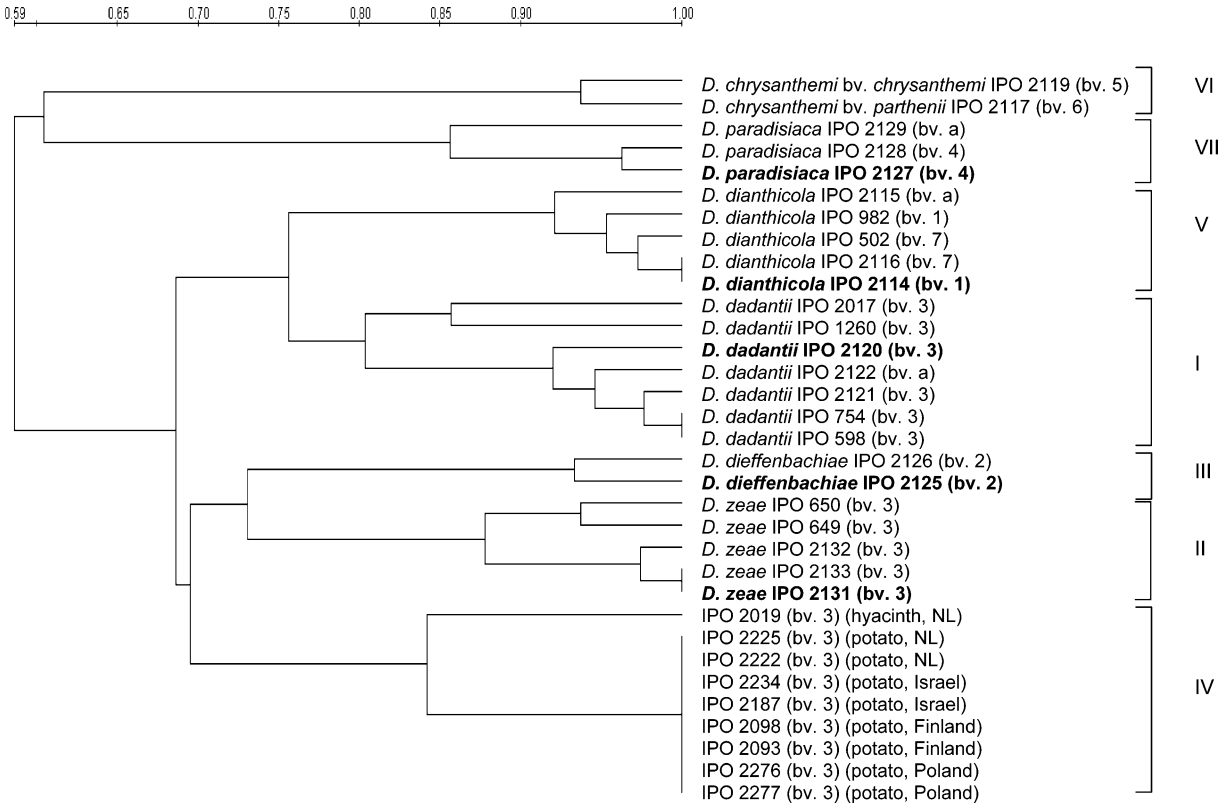


Fig. 4 Phenogram of REP-PCR patterns constructed using UPGMA (unweighted pair-group method using arithmetic means) tree building method. Type strains are indicated in bold. Clades, indicated in Roman numerals, correspond with those described in Table 1

hybridisation studies, were also clustered in six distinct groups on the basis of *dnaX* sequence analysis.

Although 16S rDNA sequence analysis is commonly used for the identification of bacterial species and phylogenetic studies, this gene was found to have a relatively poor ability to predict genome relatedness at (sub)genus level (Zeigler 2003). In line with these observations, the six different named *Dickeya* species could not be differentiated on the basis of 16S rDNA sequence analysis. Nevertheless, on the basis of 16S rDNA sequences, the new potato strains within the new biovar 3 clade together with the hyacinth strain were clustered separately from other *Dickeya* species.

The new biovar 3 clade of potato strains could also be distinguished from other *Dickeya* species using REP-PCR genomic fingerprinting. Results showed that in REP-PCR the biovar 3 hyacinth strain was similar, but not identical to the potato strains. REP-PCR followed by cluster analysis enabled classification of *Dickeya* reference strains into species similar as *dnaX* sequence analysis, showing the value of this technique for phylogenetic analysis of *Dickeya* species. Using the same technique various *Xanthomonas* spp. were successfully classified previously (Louws et al. 1992, 1994, 1995; Vauterin et al. 1995; Vera Cruz et al. 1995; Opgenorth et al. 1996). The new biovar 3 clade of potato strains reacted identically in biochemical assays, supporting the hypothesis that strains were from clonal origin. The clade only differed from other biovar 3 strains by a weaker growth at 39°C. Weak utilisation of β-gentiobiose, was not a distinguishing feature since biovar 3 strains are variable with respect to utilisation of this substrate. Results from the biochemical assays on reference strains, conducted in microtiter plates, agreed largely with those expected according to Ngwira and Samson (1990) and Palacio-Bielsa et al. (2006). For biovar 1 and 7 strains, however, no assimilation of inulin was observed. Cother et al. (1992) also described *D. dianthicola* strains negative for inulin. Furthermore, Samson et al. (2005) described 12% of the phenon 5 *D. dianthicola*

strains negative for inulin. Therefore, inulin assimilation seems not to be a reliable feature to distinguish *D. dianthicola* from other *Dickeya* species.

On the basis of the multilocus sequence analysis, including *dnaX* and 16S rDNA sequences, rep-PCR analysis, and the biovar determination, we conclude that the group of recently isolated biovar 3 potato strains belongs to a new clade distinctive from the six *Dickeya* species described by Samson et al. (2005). It may therefore constitute a new species. The new biovar 3 group of potato strains included two Finnish strains formerly characterised by Laurila et al. (2008) using ITS sequences and designated as divergent group I strains. In their studies, two groups of *Dickeya* spp. were found in potato (groups I and II). The 16S rDNA sequences and the growth at 39°C suggested that strains in group II were *D. dianthicola*. The group I strains in the 16S rDNA tree were clearly different from *D. dianthicola* strains and the previously characterised *Dickeya* species and were able to grow to single colonies at 39°C. The ITS sequences of these strains were identical and formed a divergent clade. The group I strains were, on average, more virulent in greenhouse experiments and potato tuber slice assays, but less so in a field experiment with vacuum-infiltrated tubers, although a high variation between strains was found. In our studies, strain IPO 2093, IPO 2095 and IPO 2098, belonging to the Finnish group I belonged to the new biovar 3 clade.

Most European potato strains isolated before 2000 belonged to *D. dianthicola* (biovars 1 and 7) according to biovar determinations and *dnaX* sequence analysis. Genetically they formed a relatively homogeneous group, although some sequence variation and differences in Rep-PCR analysis were found. As an exception, two potato strains from Germany isolated before 1990 were biochemically classified into biovar 2 and 3, respectively. The *dnaX* sequence cluster analysis grouped these strains into *D. dieffenbachiae* and *D. dadantii*, associated with biovar 2 and 3, respectively. This is the first time that *D. dieffenbachiae* was described in association with potato.

The new biovar 3 clade is possibly spread via Dutch seed potatoes. The strains from Israel were isolated from blackleg-diseased plants raised from Dutch seed (Tsror et al. 2008). Also in Poland (Sławiak et al. 2009) and Finland frequently Dutch seed is used for potato production. The new biovar 3 strains seem to possess epidemiological features, which makes them successful as a potato pathogen as it is found in countries with different climates and in different cultivars. The strains have a higher growth optimum than *D. dianthicola* and growth may therefore be particularly favoured at high temperature conditions. The relatively high disease expression in the greenhouse experiments in Finland supports this idea (Laurila et al. 2008). The strains may have been introduced in seed potatoes by the use of contaminated surface water for irrigation. Surface water contaminated with *Dickeya* and *Pectobacterium* has been frequently found (Pérombelon and Hyman 1987, Cappaert et al. 1988; Persson 1991; Cother et al. 1992; Norman et al. 2003). It has been speculated that contaminated surface water can be a primary source of infection for clean potato stocks (Cappaert et al. 1988; Laurila et al. 2008).

Biovar 3 potato strains described in this study, may originate from the ornamental crops which can host *Dickeya* spp. These crops are grown on a large scale in the Netherlands. Indeed, among many strains from ornamental plants tested, a *Dickeya* strain from hyacinth was very similar to the biovar 3 potato strains, although not identical. It may be that a similar strain was introduced in seed potato production before 1995, at the time that the use of surface water for irrigation in the Netherlands was not yet prohibited due to the presence of *Ralstonia solancearum*.

The new group should be further studied for epidemiological features, including host specificity, survival and dissemination. It should also be included in test programmes for screening potato cultivars for resistance to *Dickeya*.

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