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Phenotypic diversity, host range and molecular phylogeny of *Dickeya* isolates from Spain

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Abstract Six *Dickeya* spp. strains representative of a larger group of bacteria isolated from potato, onion and irrigation water in Spain between years 2003–2005, were characterised by biochemical, serological, molecular and pathogenicity assays. Biochemical and serological differences, as well as pathogenic behaviour in host range and virulence levels, were observed among the strains. They were classified into biovars 3 and 6. Phylogenetic analysis and comparison of the isolates with type strains of *Dickeya* species characterised to date were performed using concatenated partial sequences of the housekeeping genes *gapA* and *mdh*. One of the Spanish strains was identified as *D. dieffenbachiae*, whereas the other ones did not fit clearly into the previously described six *Dickeya* species, and may therefore constitute novel species. Isolation of dissimilar pathogenic strains in different rivers and irrigation water sources supports the idea that *Dickeya* species is commonly present in such an environment, and contaminated water is a potential source of inoculum for the disease in different crops.

Keywords *Dickeya* species · Genetic relationship · Housekeeping genes · Pathogenicity

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Introduction

Dickeya sp. (Samson et al. 2005) (formerly *Erwinia chrysanthemi*, Burkholder et al. 1953) is a Gram-negative plant pathogenic bacterium, belonging to the *Enterobacteriaceae* family, characterised by the production of large quantities of extracellular pectic enzymes. Variability within the former *Erwinia chrysanthemi* is large and the strains were divided into nine biovars (Ngwira and Samson 1990) and six species (Samson et al. 2005). The overall diversity of isolates formerly included in *E. chrysanthemi sensu stricto* was evident from using different methods, such as PCR amplification and sequencing or restriction fragment length polymorphism (RFLP) of 16S or 16S–-23S rDNA intergenic spacer region (Toth et al. 2001; Laurila et al. 2008), RFLP of *recA*

gene fragments (Waleron et al. 2002), amplified fragment length polymorphism (AFLP) fingerprinting (Avrova et al. 2002), multilocus phylogenetic analysis (Ma et al. 2007), rep-PCR genomic fingerprinting and *dnaX* and 16S rDNA phylogenetic analysis (Slawiak et al. 2009).

Dickeya species cause soft rot and wilting diseases of a wide range of host plants, including many cultivated plants. The bacterium is increasingly being recognised as an important pathogen of potato and it has been reported in many countries under different climatic conditions, such as semi-arid areas, tropical regions, temperate and cool-temperate regions (Palacio-Bielsa et al. 2006; Laurila et al. 2008). *Dickeya* strains have been also isolated from rivers and irrigation water sources in many countries (Laurila et al. 2008 and references therein).

The goal of the present study was to investigate a diversity of Spanish *Dickeya* strains in regard to their phenotypic, genetic, pathogenicic and host range characteristics, and analyze their genetic relationships.

Materials and methods

Isolation of bacterial strains

Diseased potato plants, showing wilt and desiccation of the top leaves and necrosis of the stem pith, were collected from commercial crops in Teruel (Spain). Small pieces of diseased stem tissue were dissected at the lesion margin and crushed in sterile distilled water. The suspensions were streaked on modified crystal violet pectate (CVP) selective medium (Pérombelon and Burnett 1991) and incubated at 20°C and 27°C for 48–72 h for facilitating the isolation of *Pectobacterium atrosepticum, Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya* spp.

Onion plants showing soft rot symptoms at the base of central leaves and inner layers of bulbs were collected in fields with drip irrigation in Zaragoza (Spain). Isolations from diseased tissues were performed as indicated above.

Water samples (100 ml) were recovered in different geographical areas. Samples were collected at 10 cm below the surface directly into sterile bottles and maintained at 4°C until processing, within the following 24 h. Water samples (60 ml) were combined with 60 ml of Doble-PEM enrichment medium (Burr and Schroth 1977) and incubated at 25°C for 48–72 h in anaerobic conditions. Serial dilutions of enriched samples (20 µl) were streaked on modified CVP selective medium and incubated as described above.

Characterisation of isolates

Single colonies obtained on CVP medium were purified and isolates from various hosts and locations studied. After initial biochemical, physiological and biovar determination, a smaller number of representative strains were selected for further analysis of the bacteria obtained from the different environments. Type strains of the six *Dickeya* species (Samson et al. 2005), as well as *D. dadantii* 3937 strain from the Collection Française de Bactéries Phytopathogènes (Angers, France) (CFBP), and one strain of *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* were included for comparison in biochemical, physiological, molecular and serological tests. All the strains were maintained at −80°C on 30% (*v*/*v*) glycerol, and 48 h-old cultures on nutrient agar (NA) (Difco) were used for the different tests.

Biochemical and physiological identification of isolates

The isolates were identified by standard bacteriological methods based on Cother and Sivasithamparam (1983). The tests performed were Gram reaction; oxidase activity; glucose metabolism; pectate degradation in Sutton´s medium; production of phosphatase; indole production from tryptophan; gelatine hydrolysis; production of reduction substances from sucrose; production of acid from α-methylglucoside and threalose; malonate utilisation; sensitivity to erythromycin (15 µg); growth at 37°C, determined after 24 h in nutrient broth (NB, Difco); salt tolerance, checked after 48 h growth in NB with 5 gl^{-1} NaCl.

PCR identification

Bacterial suspensions in sterile distilled water (10^8 cells ml^{-1}) were prepared from 48 h-old cultures on NA. Genomic DNA was extracted and purified using an Easy-DNA™ kit as described by the manufacturer (Invitrogen), quantified using a

NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific) and used as template. PCR was performed in 50 µl reaction volumes containing 100 ng of target DNA; 1 U of Taq DNA polymerase (Sigma-Aldrich); 5 µl 10× reaction buffer; 1.5 mM $MgCl_2$; 100 µM deoxynucleotide triphosphates and 5 pmol of *Dickeya*-specific primers of the pectate lyase gene cluster, *pelADE* (Nassar et al. 1996). DNA amplification was performed using a MJ Research, Inc. PTC-100 thermo-cycler, with initial denaturation at 94°C for 4 min, followed by 40 cycles consisting of incubations for 30 s at 94°C and 1 min at 72°C, and a final extension at 72°C for 10 min. Amplified fragments were run on a 2% agarose gel and visualised under UV light following ethidium bromide staining.

Serological identification

Enzyme-linked immunosorbent assay (ELISA) was performed in microplates (NuncMaxiSorp™ Brand products) (two wells per strain), using commercial polyclonal antibodies (LOEWE Phytodiagnostica; reference number 07146) and monoclonal antibodies (Agdia Incorporated; reference number CAB 73300), according to the manufacturer's instructions. The absorbance was measured with an automatic reader at 405 nm and, after 1 h, A_{405} values greater than twice the mean of negative controls were considered to be positive.

Classification of *Dickeya* isolates into biovars

Ngwira and Samson (1990) tests for biovar classification were used. Biovar classification was performed by the microtitre system described by Palacio-Bielsa et al. (2006). Tests were repeated thrice in separated microplates and experiments. In all cases, *D. dianthicola* reference strains CFBP 1888 and CFBP 2015, biovar 1 and biovar 7, respectively (Samson et al. 2005) were used as controls.

Host range and virulence assays

Potato (*Solanum tuberosum*) (cv. Agria), onion (*Allium cepa*) (cv. Fuentes), chicory (*Cichorium* sp.), African violet (*Saintpaulia ionantha*) and maize (*Zea mays*) hybrid NK-Arma (Syngenta) were used as test hosts.

Potato Tubers were inoculated with 50 µl of aqueous suspensions (10^6 cells ml^{-1}) of 24-h cultures grown on NA plates by inserting a sterile plastic micropipettor tip at a constant depth of 1.5 cm, as described elsewhere (Maggiorani Valecillos et al. 2006). The tubers were incubated in a moist chamber at 28°C for 48 h. Afterwards, tubers were sliced at the inoculation point. Two independently replicated assays were performed and seven potato tubers were inoculated at two locations in each experiment.

Onion Bacterial inocula were prepared as described above. Aseptically detached-scale pieces from mature onion bulbs were inoculated (Clark and Hale 1993), by inserting in their centre a plastic micropipettor tip with 10 µl of inoculum (10^8 cells ml^{-1}). Onion scales were incubated in a moist chamber at 28°C up to 5 days. Four onion scales per experiment were inoculated and two independent assays were performed.

Chicory Bacteria were prepared by suspending 16-h cultures grown in NA medium plates. The cells were washed with 10 mM $MgCl_2$ and then resuspended in an appropriate volume of buffer to obtain the desired inoculum concentration. Chicory leaves were inoculated by introducing 10 µl of inoculum (10^6 cells ml^{-1}) into a small wound made with a sterile toothpick on the inner surface of individual leaves (Maggiorani Valecillos et al. 2006). Leaves were maintained in a moist chamber at 28°C for 24 h. Two independent experiments were performed and 10 leaves per assay were inoculated.

African violet The cells from NA plate cultures were prepared as indicated above. Two inoculations were made in leaves of *Saintpaulia* plants as described by Maggiorani Valecillos et al. (2006). Inoculations were done by introducing 100 µl of inoculum (10^5 cells ml^{-1}) with a syringe on the inner of leaves (Maggiorani Valecillos et al. 2006). The plants were incubated in a moist chamber at 28°C for 24 h.

Maize One-month-old-seedlings, grown in pots with sterile potting substrate in a growth chamber, were inoculated. Bacteria were prepared by suspending 24-h cultures grown on NA medium plate in sterile distilled water, and 1 ml of cells suspensions (10^8 cells ml^{-1}), containing 0.5% (*v*/*v*)

of Tween^R 20 (polyoxyethylene sorbitan monolaurate) as a surfactant, was poured into the leaf whorl (Hartman and Kelman 1973). Control plants were inoculated with sterile distilled water plus Tween^R 20. The plants were maintained up to 15 days at 27°C day and 22°C night temperature. Two independent experiments were performed and seven plants per assay were inoculated. Stalks were cut longitudinally and the virulence levels of the strains were estimated by their ability to induce soft-rot lesions of the apical meristem or only of the inner whorl leaves.

In all other hosts, maceration damage was reported and the virulence levels of the strains were evaluated by measuring the size (cm^2), as the product of length and width, of the lesions. Lesion area among strains for each host was compared by a one-way analysis of variance (ANOVA) using Statgraphics *plus* version 5.0 software. Data were transformed to stabilize the variance before ANOVA. Strains not causing lesions in any replication of the experiment were not included in the ANOVA. Means were compared using the Fisher´s protected least significant difference (LSD) test at the 5% significance level.

Mock-inoculated controls were always included. In all cases, isolations from inoculated hosts were performed on CVP selective medium, and colony identification was confirmed by PCR tests.

Determination of gene sequences

Bacterial suspensions in sterile distilled water were prepared from 48-h cultures on NA medium and their concentration adjusted to 10^8 cells ml^{-1}. Genomic DNA was extracted using the Easy-DNA™ Kit (Invitrogen), according to manufacturer instructions, and quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific).

Portions of *gapA* (glyceraldehydes-3-phosphatase dehydrogenase A) and *mdh* (malate dehydrogenase) housekeeping genes from all strains were amplified with primers designed to anneal to conserved motifs (Ma et al. 2007). A ca. 534-bp length band is expected for the *gapA* gene (primers gapA326F/gapA845R), whereas a ca. 563-bp is the expected size for the *mdh* gene (primers mdh86F/mdh628R) (Ma et al. 2007). A 50-µl PCR mix containing 3.75 U of AmpliTaq Gold™ DNA Polymerase (Applied Biosystems), 5 µl 10× reaction buffer, 2.5 mM $MgCl_2$, 1.6 mM deoxynucleotide triphosphates, 0.4 µM each of forward and reverse primers and 20 ng of genomic DNA template. PCR amplifications were carried out in a S1000™ Thermal cycler (Bio-Rad Laboratories, Inc.) with the following steps: a hot start at 95°C for 3 min, 30 amplification cycles of 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. Amplified DNA was detected by electrophoresis in a 1% agarose gel stained with ethidium bromide.

The PCR products obtained were cloned using the pGEM®-T Easy Vector Systems kit (Promega Corporation). Ligation reactions were performed using a 15:1 molar ratio of insert product:vector. Recombinant plasmids were purified using the Wizard® *Plus* SV Minipreps DNA Purification System kit (Promega Corporation) as described by the manufacturer. In order to verify an insert of the expected size, purified clones were digested with *Eco*RI or *Not*I restriction enzymes for *gapA* and for *mdh* genes, respectively. Digestion products were analysed by 0.7% agarose gel and visualized under UV light after staining with ethidium bromide.

Three clones for each of the genes were sequenced. The DNA templates for sequencing were then quantified using a Nanodrop spectrophotometer (Nanodrop) and prepared at 100 ng μl^{-1}. All sequences were determined in both the forward and reverse directions. DNA sequencing of both strands was done by the chain termination method on double-stranded DNA templates with an ABIprism Dye Terminator cycle sequencing kit (Perkin-Elmer) in a 377 DNA Sequencer (Perkin Elmer). Sequence data obtained in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov.Genbank) under accession numbers from GQ891959 to GQ891971 (*gapA* gene) and GQ 891972 to GQ891984 (*mdh* gene) (Tables 1 and 3).

Sequence alignments were performed at the National Center for Biotechnology Information (on-line) with the BLAST network service (Altschul et al. 1990).

Phylogenetic analysis

Sequences were aligned and manually edited with BioEdit v7.0.9 (Hall 1999) using the program CLUSTALW with the default parameters (Thompson et al. 1994). The resultant alignment was used for the

Table 1 *Dickeya* spp., *Pectobacterium atrosepticum* and *P. carotovorum* subsp. *carotovorum* strains used in this study

Strains	Other designation (s)	Host	Geographical origin and year of isolation	Reference
CFBP 7086[a]	CITA 1	*Solanum tuberosum* cv. Agria	Teruel (Spain), 2003	
CITA 2		*Solanum tuberosum* cv. Agria	Teruel (Spain), 2003	
CITA 3		*Solanum tuberosum* cv. Agria	Teruel (Spain), 2003	
CITA 4		*Solanum tuberosum* cv. Agria	Teruel (Spain), 2003	
CITA 5		*Solanum tuberosum* cv. Agria	Teruel (Spain), 2003	
CITA A-3[a]		Water	Alcanadre river, Huesca (Spain), 2005	
CFBP 7084	CITA A-6	Water	Alcanadre river, Huesca (Spain), 2005	
CITA Q-4[a]		*Allium cepa*	Zaragoza (Spain), 2005	Palacio-Bielsa et al. 2007
CITA Q-5[a]		*Allium cepa*	Zaragoza (Spain), 2005	Palacio-Bielsa et al. 2007
CFBP 7083	CITA C-29	*Allium cepa*	Zaragoza (Spain), 2005	Palacio-Bielsa et al. 2007
CITA Q-7		*Allium cepa*	Zaragoza (Spain), 2005	Palacio-Bielsa et al. 2007
CITA M-2[a]		Water	Gállego river irrigation canal, Zaragoza (Spain), 2005	
CITA B-1[a]		Water	Maize plot sprinkler irrigation, Zaragoza (Spain), 2005	
CITA B-2		Water	Maize plot sprinkler irrigation, Zaragoza (Spain), 2005	
CITA B-3		Water	Maize plot sprinkler irrigation, Zaragoza (Spain), 2005	
Dickeya type strains				
D. chrysanthemi bv. *chrysanthemi* CFBP 2048	NCPPB 402, ATCC 11663, CIP 82.99, DSM 4610, ICMP 5703, LMG 2804	*Chrysanthemum morifolium*	USA, 1958	Samson et al. 2005
D. dadantii CFBP 1269	NCPPB 898, DSM 18020, Dye D.W. EG18, 1544-66, Hayward A.C. B374	*Pelargonium capitatum*	Comoros, 1960	Samson et al. 2005
D. dianthicola CFBP 1200	NCPPB 453, ICMP 6427, DSM 18054, LMG 2485	*Dianthus caryophyllus*	UK, 1956	Samson et al. 2005
D. dieffenbachiae CFBP 2051	CFBP 1246, NCPPB 2976, CIP 109104T, DSM 18013, ICPB ED 102,	*Dieffenbachia* sp.	USA, 1957	Samson et al. 2005
D. paradisiaca CFBP 4178	NCPPB 2511, ATCC 33242, CIP 109103T, DSM 18069, LMG 2542	*Musa paradisiaca* var. dominico	Colombia, 1970	Samson et al. 2005
D. zeae CFBP 2052	NCPPB 2538, CIP 109102T, DSM 18068, ICMP 5704	*Zea mays*	USA, 1970	Samson et al. 2005
Reference strains				
D. dadantii CFBP 3855	Lemattre 3937	*Saintpaulia ionantha*	Vendée (France), 1977	Samson et al. 2005
D. dianthicola CFBP 2015		*Solanum tuberosum*	France, 1975	Samson et al. 2005
D. dianthicola CFBP 1888	Sailly I/K col2	*Solanum tuberosum*	Ille-et-Vilaine Saint Malo (France), 1978	Samson et al. 2005

Table 1 (continued)

Strains	Other designation (s)	Host	Geographical origin and year of isolation	Reference
Pectobacterium atrosepticum SCRI Eca 1001		*Solanum tuberosum*	Scotland	
P. carotovorum subsp. *carotovorum* CPV Ecc-3		*Cynara scolymus*	Zaragoza (Spain), 1994	Palacio-Bielsa et al. 2006

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[a] Strains considered as representative of bacteria obtained from their respective environments and selected for further analysis

construction of phylogenetic trees with the following methods: 1) neighbour joining using the MEGA 4 platform (Tamura et al. 2007) and 100,000 replicates for bootstrap analysis; 2) Bayesian phylogeny using MRBAYES (Ronquist and Huelsenbeck 2003) under the GTR evolution model (Posada 2008), with 100,000 replicates for bootstrap and re-sample every 100 generations; and 3) maximum likelihood using Phylogeny.fr (Dereeper et al. 2008) with 500 replicates for bootstrap.

Results

Biochemical and physiological identification of isolates as *Dickeya* sp.

Colonies from plates incubated at 27°C, which appeared on selective CVP medium with the typical pectinolytic cavities, were purified by repeated sub-culturing on NA medium, and 15 isolates were selected for initial studies. With few exceptions, the isolates were very similar and their biochemical and physiological tests results agreed with those expected for *Dickeya* sp., according to Cother and Sivasithamparam (1983). However, the *D. dianthicola* type species strain CFBP 1200 showed a negative reaction for phosphatase production test; both *D. dieffenbachiae* CFBP 2051 and *D. paradisiaca* CFBP 4178 type strains did not produce gelatin hydrolysis; onion isolate CITA Q-4 produced acid from α-methylglucoside and the water isolate CITA A-3, as well as onion isolates CITA Q-4, CITA Q-5 and CFBP 7083 showed a weakly positive reaction for the 5 gl^{-1} NaCl salt tolerance test (Table 2).

Molecular identification

The expected 420-bp PCR-amplified fragment for specific *Dickeya* primers (Nassar et al. 1996) was obtained from all the Spanish strains. Identical results were observed for the *Dickeya* species reference strains (Samson et al. 2005). As expected, no amplification was obtained for *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* reference strains (Table 3).

Serological identification

Similar results were obtained for both monoclonal and polyclonal antibodies in ELISA tests. Positive reactions were obtained with the Spanish strains selected for further analysis, CFBP 7086 (CITA 1), CITA A-3 and CITA Q-5, *D. dadantii* CFBP 3855 reference strain, as well as *Dickeya* type strains *D. dadantii* CFBP 1269, *D. chrysanthemi* CFBP 2048, *D. dieffenbachiae* CFBP 2051 and *D. dianthicola* CFBP 1200, with absorbance mean values greater than 10 times the mean values of negative controls. Conversely, antibodies did not react with onion strain CITA Q-4 and water strains CITA M-2 and CITA B-1, or with *D. paradisiaca* CFBP 4178 and *D. zeae* CFBP 2052 type strains. No cross-reactions were observed with *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* reference strains (Table 3).

Table 2 Biochemical, physiological properties and biovar identification of *Dickeya* spp., *Pectobacterium atrosepticum* and *P. carotovorum* subsp. *carotovorum* strains used in this study

Strains[a]	Biochemical and physiological tests										Biovar
	Phosphatase activity	Indol from tryptophan	Gelatine hydrolisis	Reduced substances from sucrose	Acid from α-methylglucoside	Acid from threalose	Malonate utilisation	Erythromycin sensitivity (15 μg)	Growth 37°C	Growth 5 gl^{-1} NaCl	
CFBP 7086[b]	+	+	+	–	–	–	+	+	+	–	6
CITA 2	+	+	+	–	–	–	+	+	+	–	6
CITA 3	+	+	+	–	–	–	+	+	+	–	6
CITA 4	+	+	+	–	–	–	+	+	+	–	6
CITA 5	+	+	+	–	–	–	+	+	+	–	6
CITA A-3[b]	+	+	+	–	–	–	+	+	+	+w	3
CFBP 7084	+	+	+	–	–	–	+	+	+	–	3
CITA Q-4[b]	+	+	+	–	+	–	+	+	+	+w	3
CITA Q-5[b]	+	+	+	–	–	–	+	+	+	+w	3
CFBP 7083	+	+	+	–	–	–	+	+	+	+w	3
CITA Q-7	+	+	+	–	–	–	+	+	+	–	3
CITA M-2[b]	+	+	+	–	–	–	+	+	+	–	3
CITA B-1[b]	+	+	+	–	–	–	+	+	+	–	3
CITA B-2	+	+	+	–	–	–	+	+	+	–	3
CITA B-3	+	+	+	–	–	–	+	+	+	–	3
Dickeya type strains				–	–						
D. chrysanthemi bv. *chrysanthemi*CFBP 2048	+	+	+	–	–	–	+	+	+	–	5
D. dadantii CFBP 1269		+	+	–	–	–	+	+	+	–	3
D. dianthicola CFBP 1200	–	+	+	–	–	–	+	+	+	–	1
D. dieffenbachiae CFBP 2051	+	+	–	–	–	–	+	+	+	–	2
D. paradisiaca CFBP 4178	+	+	–	–	–	–	+	+	+	–	4
D. zeae CFBP 2052	+	+	+	–	–	–	+	+	+	–	3
Reference strains											
D. dadantii CFBP 3855	+	+	+	–	–	–	+	+	+	–	3
D. dianthicola CFBP 2015	+	+	+	–	–	–	+	+	+	–	7
D. dianthicola CFBP 1888	+	+	+	–	–	–	+	+	+	–	1

Table 2 (continued)

Strains[a]	Biochemical and physiological tests										Biovar
	Phosphatase activity	Indol from tryptophan	Gelatine hydrolisis	Reduced substances from sucrose	Acid from α-methylglucoside	Acid from threalose	Malonate utilisation	Erythromycin sensitivity (15 μg)	Growth 37°C	Growth 5 gl^{-1} NaCl	
Pectobacterium atrosepticum SCRI Eca 1001	–	–	+	+	+	+	–	–	–	+	
P. carotovorum subsp. *carotovorum* CPV Ecc-3	–	–	+	–	–	+	–	–	+	+	

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w, weak reaction

[a] Gram, oxidase, glucose metabolism and pectate degradation in Sutton´s medium tests results of all the strains agreed with those expected for *Dickeya* sp., *Pectobacterium atrosepticum* and *P. carotovorum* subsp. *carotovorum*, according to Cother and Sivasithamparam (1983)

[b] Strains considered as representative of bacteria obtained from their respective environments and selected for further analysis

Classification of *Dickeya* sp. strains into biovars

Spanish strains were classified into biovar 6 (isolates CFBP 7086, CITA 2, CITA 3, CITA 4 and CITA 5) and biovar 3 (isolates CITA A-3, CFBP 7084, CITA Q-4, CITA Q-5, CFBP 7083, CTA Q-7, CITA M-2, CITA B-1, CITA B-2 and CITA B-3), according to Ngwira and Samson (1990). In all cases, results agreed with those expected for the *Dickeya* reference strains CFBP 2015 and CFBP 1888 included as controls (Table 2).

Host range and virulence tests

Results of the virulence analysis showed statistically significant differences in the symptom severity of the selected 13 *Dickeya* spp. strains among the five hosts (Table 3). No lesions developed on uninoculated controls (data not shown).

In general, strains CITA A-3, CITA Q-4 and CITA Q-5 were the most aggressive on potato tubers, onion bulbs and *Saintpaulia* leaves. In chicory, the most virulent strains were CITA Q-4, CITA Q-5 and CFBP 2051. Several strains did not cause disease symptoms at all on certain hosts. Strains CFBP 3855, CFBP 2048, CFBP 1269 and CFBP 1200 did not induce disease on onion; the latter two strains did not cause disease on *Saintpaulia*. On chicory, strains CITA M-2 and CITA B-1 were non-pathogenic (Table 3).

All strains were pathogenic to maize plants, although variations in symptom severity were observed among the strains. Strains CFBP 7086, CITA B-1 and CFBP 1200 were highly aggressive, spreading internally and inducing soft-rotting of the whole apical meristem and other internal tissues (observed by longitudinal cuttings), and plants collapsed within 5–7 days after inoculation. Moderately aggressive strains CITA A-3, CITA M-2, as well as CFBP 4178 and CFBP 2052, induced rotting to a lesser extent and only affected the upper part of the apical meristem. Externally, wilting of the leaves emerging from the whorl was observed and breaking of the apical dominance resulted in shoot growth from lower axillary buds. Strains CITA Q-4, CITA Q-5, CFBP 2048, CFBP 1269, CFBP 2051 and CFBP 3855 were weakly aggressive and only induced necrosis and wilting symptoms of the leaves emerging from the whorl (Table 3).

Pectolytic colonies were obtained on CVP medium from each diseased host, whereas no cavity-forming colonies were observed from uninoculated controls.

Table 3 PCR, ELISA, hosts range, virulence levels and GenBank accesion numbers of the *Dickeya* spp. strains studied

Strains	PCR	ELISA	Inoculation tests hosts and virulence levels[a]					GenBank accesion numbers *gapA* / *mdh* genes
			Potato	Onion	Chicory	African violet	Maize	
CFBP 7086 (CITA 1)	+	+	3.09 de	2.74 c	0.49 bc	0.84 bc	+++	GQ891959 / GQ891972
CITA A-3	+	+	5.87 f	19.46 d	0.37 a	1.59 d	++	GQ891960 / GQ891973
CITA Q-4	+	–	8.42 g	44.75 e	1.83 d	5.58 e	+	GQ891961 /GQ891974
CITA Q-5	+	+	5.74 f	27.66 e	1.58 d	6.05 e	+	GQ891962 / GQ891975
CITA M-2	+	–	0.21 a	0.83 ab	0.00 [b]	0.13 a	++	GQ891963 / GQ891976
CITA B-1	+	–	0.73 ab	2.70 bc	0.00 [b]	0.42 ab	+++	GQ891964 / GQ891977
Dickeya type strains								
D. chrysanthemi bv. *chrysanthemi* CFBP 2048	+	+	2.27 cd	0.00 [b]	0.66 c	0.54 abc	+	GQ891967 / GQ891980
D. dadantii CFBP 1269	+	+	2.08 c	0.00 [b]	0.52 bc	0.00 [b]	+	GQ891966 / GQ891979
D. dianthicola CFBP 1200	+	+	0.73 ab	0.00 [b]	0.47 ab	0.00 [b]	+++	GQ891971 / GQ891984
D. dieffenbachiae CFBP 2051	+	+	3.35 de	0.04 a	1.74 d	0.20 ab	+	GQ891968 / GQ 891981
D. paradisiaca CFBP 4178	+	–	0.46 a	0.14 a	0.66 c	2.09 a	++	GQ891969 /GQ891982
D. zeae CFBP 2052	+	–	2.04 c	0.12 a	0.96 c	0.28 ab	++	GQ891970 / GQ891983
Reference strain								
D. dadantii CFBP 3855	+	+	1.44 b	0.00 [b]	0.75 c	1.04 c	+	GQ891965 / GQ891978
D. dianthicola CFBP 2015	+	+	Nd	Nd	Nd	Nd	Nd	
D. dianthicola CFBP 1888	+	+	Nd	Nd	Nd	Nd	Nd	
Pectobacterium atrosepticum SCRI Eca 1001	–	–	Nd	Nd	Nd	Nd	Nd	
P. carotovorum subsp. *carotovorum* CPV Ecc-3	–	–	Nd	Nd	Nd	Nd	Nd	

For each host, values followed by the same letter do not differ significantly (α=0.05) as determined by Fisher's protected LSD test

Maize plants virulence levels are referred to the ability of rot inducing of the apical meristem or only of the inner whorl leaves. (+++) Highly aggressive, (++) moderately aggressive, (+) weakly aggressive, Nd, not determined

CITA Centro de Investigación y Tecnología Agroalimentaria de Aragón, Zaragoza, Spain. *CFBP* Collection Française de Bactéries Phytopathogènes, Angers, France. *SCRI* Scottish Crop Research Institute, Dundee, Scotland, UK; *CPV* Centro de Protección Vegetal, Zaragoza, Spain

[a] Potato, onion, chicory and African violet: virulence levels are referred as the product of length and width size (cm^2) of the lesions in two independent assays

[b] not included in the ANOVA

Purified single pectolytic colonies yielded positive PCR amplification with specific *Dickeya* primers (Nassar et al. 1996).

Phylogenetic analysis

To determine the phylogeny of the *Dickeya* strains isolated in this study, two housekeeping genes (*gapA* and *mdh*) were cloned and sequenced. The *gapA* (glyceraldehydes-3-phosphatase dehydrogenase A), and *mdh* (malate dehydrogenase) housekeeping genes were chosen because they are present in most enterobacterias and their products are involved in diverse aspects of bacterial metabolism. Preliminary analysis suggested that there would be enough sequence diversity in these genes to allow us to reconstruct bacterial phylogenies, and these genes are not clustered in the genome. Moreover, these genes have been previously used for phylogenetic analysis of enteric bacteria (Boyd et al. 1994; Brown et al. 2000; Ma et al. 2007).

Phylogenetic trees were constructed from concatenated partial *gapA* and *mdh* sequences using three different algorithms: neighbour joining, Bayesian inference and maximum likelihood (Fig. 1). In parallel, we performed identical phylogenetic analysis using the *gapA* and *mdh* sequences independently. The topology of the resulting trees was identical.

We used the sequences of the closely related species, *P. atrosepticum,* as the outgroup, obtained from the public database ASAP (http://asap.ahabs.wisc.edu/asap/home.php). The results of the phylogenetic analyses showed that topology of the three trees obtained with each method was identical and bootstrap replications (in most cases) were higher than 98%, suggesting that their structures are robust and have a high level of confidence.

Regarding the field strains studied in this work, CITA Q-4 and CITA Q-5 were phylogenetically related and clearly belong to one or two novel *Dickeya* species. Similarly, although the strains CITA M-2 and CITA B-1 were phylogenetically close to *D. zeae*, they could belong to one or two novel species of *Dickeya* (Fig. 1). The strain CITA A-3 was closely related to the strain CFBP 2051, belonging to the species *D. dieffenbachiae*, and the strain CFBP 7086 was related to the strain representing the species *D. chysanthemi* (CFBP 2048) (Fig. 1).

Fig. 1 Evolutionary relationships of 13 *Dickeya* strains. The evolutionary history was inferred using 3 different methods, from the concatenated nucleotide sequences of genes *gapA* and *mdh*. The sequence from *Pectobacterium atrosepticum* was included as an out-group. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap tests is shown next to branches. The trees are drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. **a** Tree obtained by the neighbour joining method using MEGA 4 (Tamura et al. 2007). **b** Tree obtained by MRBAYES method (Ronquist and Huelsenbeck 2003). **c** Tree obtained by the maximum likelihood method using Phylogeny.fr (Dereeper et al. 2008)

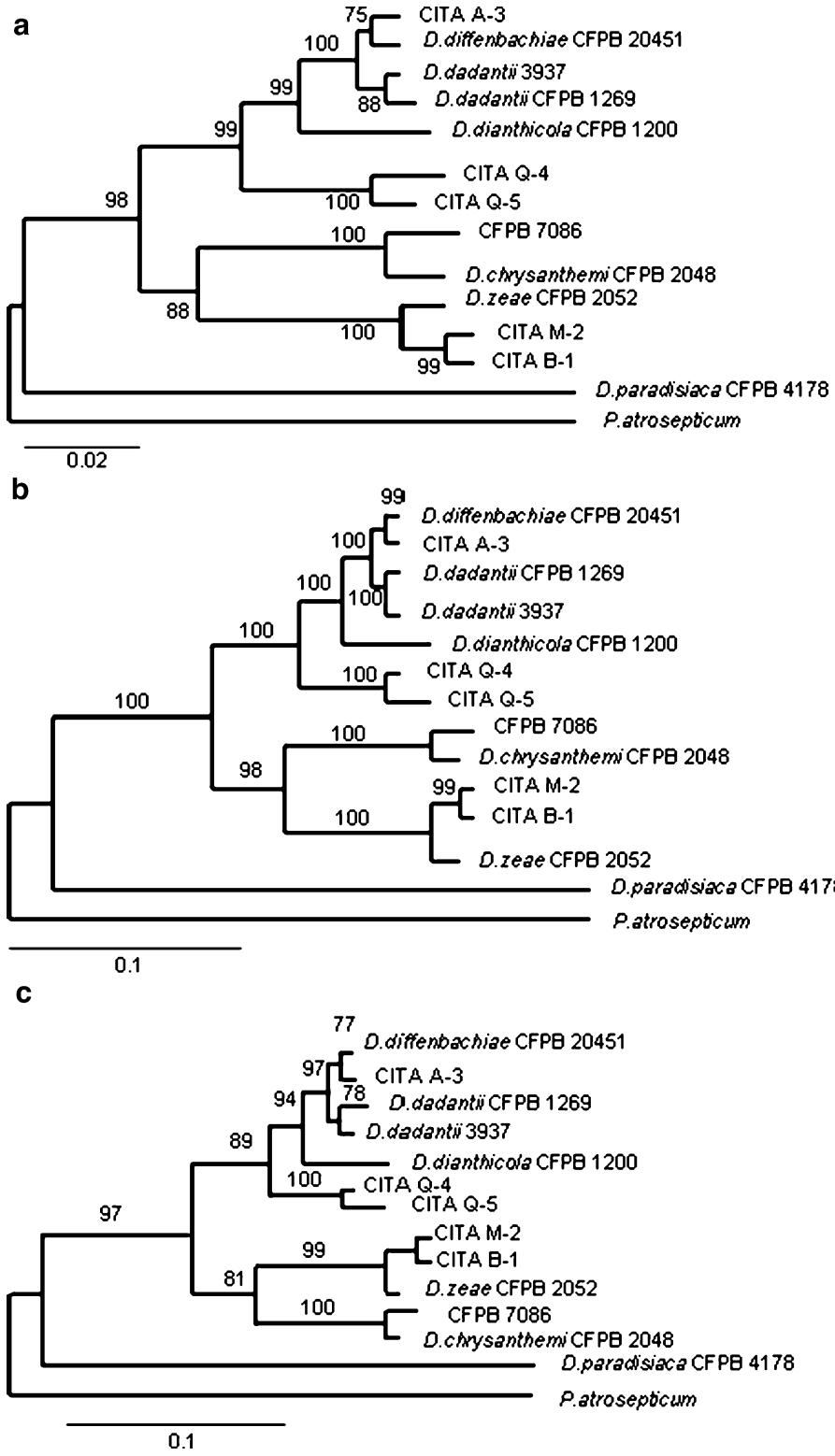
Discussion

The biochemical, physiological and PCR tests confirmed the identification of all Spanish isolates as genus *Dickeya* (formerly *Erwinia chrysanthemi*). The biochemical and physiological tests revealed some differences among isolates. Phenotypic differences, including those observed in the present work, have been previously reported by numerous authors in *Dickeya* strains isolated from different hosts. As indicated by Cother and Sivasithamparam (1983), a new strain may present slight variations in biochemical or physiological traits from the species standard description and still be considered as a valid member of such species.

Only two (biovars 3 and 6) of the nine biovars described by Ngwira and Samson (1990) were found among *Dickeya* strains from potato, onion and irrigation water sources obtained from different locations and years in Spain. As far as we know, in European countries, biovar 6 potato strains have been only previously reported in Spain (Palacio-Bielsa et al. 2006). Conversely, biovar 3 *Dickeya* strains from water have also been found in Australia (Cother et al. 1992). There is no available information about biovars of *Dickeya* strains from onion, probably because of the limited studies performed on this host.

Positive reactions were obtained with *Dickeya* type species belonging to O-serogroup 1 with both monoclonal and polyclonal commercial antisera in ELISA tests, whereas no reaction was produced with those type strains belonging to O-serogroups 3 and 6 (Samson et al. 2005). With regard to Spanish strains, no reactions were obtained with strains CITA Q-4, CITA M-2 and CITA B-1. Since commercial antibodies utilised in the present study were produced against O-serogroup 1, these strains could therefore belong to different serogroups. In accordance with other authors (e.g. Dickey et al. 1984), we also have not found a correlation between serological reactions and biovar or original hosts of the *Dickeya* strains analysed.

In this work, phylogenetic analyses were performed using concatenated partial sequences of housekeeping genes, *gapA* and *mdh*, for field isolates and a collection of strains belonging to the genus *Dickeya*. Phylogenetic analyses based on housekeeping genes in *Enterobacteriaceae* have been proven to be more reliable than the commonly used 16S rDNA (Ma et al. 2007; Young and Park 2007; Brown et al. 2000). In fact, the 16S sequence has been reported to lead to inconsistent results in some cases when used for phylogenetic analysis (Ma et al. 2007; Laurila et al. 2008; Naum et al. 2008; Slawiak et al. 2009). For example, Slawiak et al. (2009) after analyzing several species of *Dickeya* using both *dnaX* and 16S rDNA concluded that the former resulted in more robust


a
75
100
99
88
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98
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88
100
99
CITA A-3
D.diffenbachiae CFPB 20451
D.dadantii 3937
D.dadantii CFPB 1269
D.dianthicola CFPB 1200
CITA Q-4
CITA Q-5
CFPB 7086
D.chrysanthemi CFPB 2048
D.zeae CFPB 2052
CITA M-2
CITA B-1
D.paradisiaca CFPB 4178
P.atrosepticum
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D.diffenbachiae CFPB 20451
CITA A-3
D.dadantii CFPB 1269
D.dadantii 3937
D.dianthicola CFPB 1200
CITA Q-4
CITA Q-5
CFPB 7086
D.chrysanthemi CFPB 2048
CITA M-2
CITA B-1
D.zeae CFPB 2052
D.paradisiaca CFPB 4178
P.atrosepticum
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D.diffenbachiae CFPB 20451
CITA A-3
D.dadantii CFPB 1269
D.dadantii 3937
D.dianthicola CFPB 1200
CITA Q-4
CITA Q-5
CITA M-2
CITA B-1
D.zeae CFPB 2052
CFPB 7086
D.chrysanthemi CFPB 2048
D.paradisiaca CFPB 4178
P.atrosepticum
0.1

phylogenies. Moreover, we previously realized that in the genome of *D. dadantii* 3937 there are at least seven copies of 16S rDNA (http://asap.ahabs.wisc.edu/asap/home.php) and that there is a certain amount of sequence variability among them, which could easily introduce noise into the phylogenetic analysis.

We used three different types of algorithms to construct phylogenies: neighbour joining, maximum likelihood and Bayesian analysis, and the topologies of the trees were identical. This fact, in addition to the high bootstrap values of the nodes, gives confidence to the phylogenetic relationships found. Our results fit well with those reported by Slawiak et al. (2009), although they contrasted with those previously reported by Samson et al. (2005). A possible explanation for this discrepancy may be related to the above mentioned use of 16S sequences vs. housekeeping genes. Our tree differs with respect to that reported by Slawiak et al. (2009) only in the position of *D. zeae*. It is likely that this discrepancy is due to the use of sequences from different genes. In our case, we utilised the concatenated partial sequences of genes *gapA* and *mdh*, while Slawiak et al. (2009) used the sequence of the gene *dnaX*.

In agreement with other authors (Laurila et al. 2008; Slawiak et al. 2009), our results indicate that there is large genetic variation within the genus *Dickeya*, and some novel strains do not fit into the six species characterised to date (Samson et al. 2005). Clearly, field strains CITA Q-4, CITA Q-5, CITA M-2 and CITA B-1 belong to (at least) two novel species. Also, it is not yet clear whether strain CFBP 7086 belongs to *D. chrysanthemi* or whether it should be placed in a novel species.

As far as we know, *Dickeya* strains from onion recently isolated in Spain (Palacio-Bielsa et al. 2007) constitute the only report on this host in Europe. As suggested by Laurila et al. (2008), it is possible that some *Dickeya* strains, which did not cluster well with the six known *Dickeya* species (Samson et al. 2005), have recently adapted to a given host or are characteristic of strains in certain climatic zones, and therefore have not been characterised earlier. Both CITA Q-4 and CITA Q-5 strains shared many biochemical and physiological characteristics, except the α-methylglucoside and ELISA assays. Also, both strains showed a similar pathogenic behaviour in several hosts, being very aggressive in all cases except in maize. Only in potato, was the virulence of strain CITA Q-4 statistically different to strain CITA Q-5. Water strains CITA M-2 and CITA B-1 were closely related and clustered with *D. zeae*, but only strain CITA B-1 was highly aggressive in this crop according to our virulence assays. Overall, the physiological and biochemical profile of these strains were identical and pathogenic profiles were similar. Potato strain CFBP 7086 (CITA 1) was phylogenetically related to the *D. chrysanthemi* type strain and it shared many biochemical features with it; however, the strains differed in virulence on onion and maize.

In accordance with our phylogenetic analysis, water strain CITA A-3 was closely related to the *D. dieffenbiachiae* type strain (Samson et al. 2005). However, both strains differed in several biochemical tests and, moreover, showed a very different level of aggressiveness on most of the hosts.

It is known that freshly isolated strains are more likely to have retained pathogenicity and aggressiveness than those that have been kept a long time in culture collections. However, in our pathogenicity tests, the aggressiveness or even failure to cause disease by certain strains differed among hosts and also varied among strains that were isolated almost at the same time. Therefore the observed differences do not appear to be related to the loss of aggressiveness resulting from long-term preservation.

Overall, we did not find a clear concordance between biochemical tests, phylogenetic relationships and pathogenic behaviour in several hosts. This is in line with the hypothesis that the virulence of this bacterium in a particular host is a truly multifactorial character and not easily deduced from the phylogenetic position of the strain. The virulence of this pathogen is due to several factors such as the pectate lyases and other hydrolytic enzymes that degrade the plant cell wall causing the maceration and eventual necrosis of plant tissues (Alfano and Collmer 1997), iron transport functions (Expert 1999) and *hrp* genes, which encode a type III secretion system (Alfano and Collmer 1997). Also, considerable attention has been paid to the role of bacterial resistance to the host environment, such as the ability to detoxify reactive oxygen species (Miguel et al. 2000), the resistance to plant antimicrobial compounds (Maggiorani Valecillos et al. 2006) or the survival at acidic pH (Llama-Palacios et al. 2005). Therefore, it could be hypothesized that the virulence of a particular strain in certain hosts could change easily (in evolutionary terms) by changes, for example,

in the expression of some pectic enzymes or the gain of a new multidrug resistance gene by horizontal transfer.

In the last years, the incidence of *Dickeya* on potato crops has increased in Spain (Palacio-Bielsa et al. 2006). The bacterium has also been isolated from diseased maize and onion crops in newly developed irrigation land in Aragón (Spain), which have been transformed from dry crop farming areas where *Dickeya* hosts had not been previously cultivated (Palacio-Bielsa and Cambra unpublished). The isolation of pathogenic *Dickeya* strains from rivers and irrigation water sources in the present study is considered to be circumstantial evidence that water might have been a primary source of inoculum for the disease. In fact, *Dickeya* strains CITA B-1, CITA B-2 and CITA B-3 were isolated from a pump in a maize plot irrigated with centre pivot overhead sprinklers and these strains were identical to those isolated from diseased plants (data not shown). Furthermore, the increasing incidence of diseases caused by *Dickeya* spp. in these irrigated areas must be considered as further evidence that water is an important source of *Dickeya* inoculum.

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