Phylogenetic study of Japanese Dickeya spp. and development of new rapid identification methods using PCR–RFLP

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BACTERIAL AND PHYTOPLASMA DISEASES

Phylogenetic study of Japanese *Dickeya* spp. and development of new rapid identification methods using PCR–RFLP

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Abstract Forty-one representative Japanese *Dickeya* spp. (*Erwinia chrysanthemi*) strains isolated from 24 plants in Japan were investigated using multilocus sequence analysis of *recA*, *dnaX*, *rpoD*, *gyrB* and 16S rDNA; PCR–RFLP (restriction fragment length polymorphism) of *recA*, *rpoD* and *gyrB* genes; PCR genomic fingerprinting; and biochemical tests. Based on the *recA*, *dnaX, rpoD, gyrB* and 16S rDNA sequences and PCR genomic fingerprinting, the strains were essentially divided into six groups (I–VI). Group I corresponded to *D. chrysanthemi*, group II corresponded to *D. dadantii*, group III to *D. dianthicola* and group IV to *D. zeae*. Meanwhile, group V and group VI could not be assigned to any existing *Dickeya* species, and they were deduced to be two putative new species. The PCR–RFLP analysis of *gyrB, rpoD* and *recA* clearly differentiated the six groups of *Dickeya* strains. From the results of the biochemical tests, the strains were assigned to biovars 1, 3, 5, 8 and 9; only one strain (SUPP 2525) was not assignable to the existing biovars. We also showed that the PCR–RFLP analysis of *rpoD*, *gyrB* and *recA* can be used as a rapid technique to identify Japanese *Dickeya* strains.

Keywords Biochemical assays · *Dickeya* strains · Genomic fingerprinting · MLSA (multilocus sequence analysis) · PCR–RFLP

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Introduction

The genus *Erwinia* (family *Enterobacteriaceae*) was first established by Winslow et al. (1917), named after an American bacterial plant pathologist, Erwin F. Smith, to group the gram-negative and peritrichously flagellate plant pathogenic bacteria. Since then, many taxonomic proposals concerning the genus have been published, and many researchers have considered the genus to be heterogeneous, comprising several groups of species such as "true erwinias", the phytopathogenic enterobacteria causing dry necrosis or wilt diseases of plants, and "soft rot erwinias", the pectolytic soft rot enterobacteria (Starr and Chatterjee 1972). Waldee 1945 proposed that the genus *Erwinia* should be restricted to the non-pectolytic plant pathogenic enterobacteria and that the pectolytic enterobacteria should be moved into a new genus, namely *Pectobacterium*. However, the genus name *Pectobacterium* was not fully accepted by other phytobacteriologists, and *Erwinia* continued to be commonly used as the name of the genus (Starr and Chatterjee 1972).

On the basis of biochemical characteristics, Dye divided the genus *Erwinia* into four groups, i.e., an "*amylovora*" group (the "true erwinia") (Dye 1968); a "*carotovora*" group (pectolytic species, which were also known as the soft rot erwinias) (Dye 1969a); a "*herbicola*" group (yellow-pigment-producing species) (Dye 1969b) and a group

consisting of "atypical" erwinias (Dye 1969c). Dye (1968, 1969a, b, c) recommended that the genus *Erwinia* should be limited to five species with their varieties; *E. amylovora* (var. *amylovora*, var. *salicis*, var. *traceiphila*, var. *quercina*, var. *nigrifluens*, var. *rubrifaciens*); *E. herbicola* (var. *herbicola*, var. *ananas*); *E. uredovorus*; *E. stewartii* and *E. carotovora* (var. *carotovora*, var. *atroseptica*, var. *rhapontici*, var. *chrysanthemi*, var. *cypripedii*). In line with the proposal of Dye (1969a), in the chapter on the genus *Erwinia* in the 8th edition of *Bergey's Manual of Determinative Bacteriology*, the "*carotovora*" group (the soft rot erwinias) was divided into five species and subspecies (*E. cypripedii*, *E. rhapontici*, *E. carotovora* var. *atroseptica*, *E. carotovora* var. *carotovora* and *Erwinia chrysanthemi*) (Lelliot 1974).

E. chrysanthemi has been reported as one of the most important bacterial pathogens causing soft rot diseases in many cultivated crops and ornamentals both in tropical and subtropical regions (Dickey 1979; Elphinstone 1987; Ma et al. 2007; Perombelon and Kelman 1980). Based on pathological and biochemical properties, *E. chrysanthemi* had been divided into five pathovars, i.e., pv. *chrysanthemi*, pv. *zeae*, pv. *dieffenbachiae*, pv. *parthenii* (Dye 1978) and pv. *dianthicola* (Dickey 1979). Later, Dickey and Victoria (1980) proposed the transfer of *E. chrysanthemi* isolated from banana to a new pathovar, pv. *paradisiaca*. On the basis of 16S rDNA sequences, Hauben et al. (1998) proposed that the pathovars of *E. chrysanthemi*, except for pv. *paradisiaca* be grouped into the revived genus name, *Pectobacterium,* as pathovars of *P. chrysanthemi* and that *E. chrysanthemi* pv. *paradisiaca* be renamed *Brenneria paradisiaca*. Based on later phenotypic tests, DNA–DNA hybridization, serology and 16S rDNA sequence analyses, the new genus *Dickeya* was proposed by Samson et al. (2005), with *P. chrysanthemi* pathovars and *B. paradisiaca* grouped into *Dickeya*, which has six species: *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca* and *D. zeae*.

Identification of plant pathogenic bacteria is the primary step in studies of bacterial plant diseases. Information on the identity and diversity of strains of pathogens prevailing in each area is very important to understand their ecology and to control the diseases they cause. This information is also essential to develop resistant varieties. Information on strain diversity is also essential for plant quarantine organizations to detect and handle invasive species of plant pathogenic bacteria.

The use of biochemical tests for erwinia identification (Dye 1968, 1969a, b, c), as well as *Dickeya* spp. (=*E. chrysanthemi*), is still widely performed. DNA analysis is also commonplace. Sequencing and phylogenetic analysis of 16S rDNA to identify and differentiate *Erwinia* species (Duarte et al. 2004; Hauben et al. 1998; Jiménez-Hidalgo et al. 2004; Kim et al. 1999; Kwon et al. 1997) and of *recA* and other genes have been reported as powerful tools to differentiate *Dickeya* spp. (Parkinson et al. 2009; Young and Park 2007). Slawiak et al. (2009) reported that sequence analysis of *dnaX* can also be used to differentiate *Dickeya* spp., and *gyrB* and *rpoD* sequence analyses have also been used to identify *Erwinia* isolates from bacterial shoot blight of pear in Japan (Matsuura et al. 2007).

Many strains of *Dickeya* spp. (=*E. chrysanthemi*) have been isolated and identified as pathogens of various plant diseases worldwide; however, their phylogenetic positions have not been fully elucidated. Based on biochemical characteristics, the *Dickeya* species (=*E. chrysanthemi*) have been grouped into six phena (phenotypic groups) by Samson et al. (2005) and nine biovars by Ngwira and Samson (1990). Phenon 1 (all the members of biovars 3 and 8) corresponds to *D. zeae* and *D. dadantii*, phenon 2 (all the members of biovar 6) to *D. chrysanthemi*, phenon 3 (all biovar 2) to *D. dieffenbachiae*, phenon 4 (biovar 5) to *D. chrysanthemi.* Phenon 5 (biovars 1, 7 and 9) corresponds to *D. dianthicola* and phenon 6 (biovar 4) to *D. paradisiaca*.

In Europe, a group of strains in biovar 3 that was isolated from potato has been reported as a new species-level group of *Dickeya* (Laurila et al. 2008; Parkinson et al. 2009; Slawiak et al. 2009). This group has been named as group I by Laurila et al. (2008) [using the 16S–23S rDNA intergenic spacer (ITS) and 16S rDNA sequence analysis], as *Dickeya* species complex unassigned clade 1 (DUC-1) by Parkinson et al. (2009) (using *recA* sequence analysis), and as clade IV by Slawiak et al. (2009) (using repetitive extragenic palindromic [REP]–PCR genomic fingerprinting, 16S rDNA and *dnaX* sequence analysis). This group is tentatively referred to as "*Dickeya solani*", but the name has not yet been formally proposed (Toth et al. 2011). Two other groups of unclassified *Dickeya* spp., have also been isolated from several hosts other than potato in Europe. Those isolated from *Phalaenopsis* sp., *Musa* sp., *Yucca* sp., *Colocasia esculanta*, *Polyscias filicifolia*, are grouped in DUC-2, and a single strain isolated from *Aglaonema* sp. is grouped in DUC-3 (Parkinson et al. 2009). However, until now, information on the diversity of *Dickeya* spp. originating from Asian countries has been limited.

In Japan, many *E. chrysanthemi* (=*Dickeya* spp.) strains have also been isolated from various plants such as potato (Tanii et al. 1971; Tominaga and Ogasawara 1979), rice (Goto 1979, 1983; Uematsu et al. 1985), corn (Takikawa and Yamashita 1982; Takeuchi and Kodama 1992), Welsh onion (Takikawa et al. 1983), carnation (Saito 1985), eggplant (Matsuda et al. 1984), Japanese pear (Umemoto and Nagai 1984; Suyama et al. 1987), setaria (foxtail millet) (Kijima 1985), taro (Sugama et al. 1986), *Phalaenopsis* (Ito et al. 1990a), *Oncidium* and *Vanda* (Ito et al. 1990b), yacon (Mizuno et al. 1993), chicory (Sakai 1995), strawberry (Yoshimatsu and Hasama 1997), *Kalanchoe* (Sakai 1997), sweet potato (Tamura et al. 1998), peach

(Kanno et al. 2002; Funakubo et al. 2010), mango (Miyahira et al. 2008) and Chinese lantern plant (*Physalis alkekengi* L.) (Yanagiya et al. 2013). At present, their taxonomic assignment as *Dickeya* has not been confirmed, and they are still identified as *E. chrysanthemi*.

Here, we investigated the species assignment of Japanese *E. chrysanthemi* isolates in the genus *Dickeya* and developed a rapid identification method for the strains using biochemical and molecular techniques. Because of the paucity of information on the *Dickeya* spp. reported in Asia, studies on these Japanese *E. chrysanthemi* (=*Dickeya* spp.) strains will provide valuable information on the diversity of *Dickeya* spp. in Asia.

Materials and methods

Bacterial strains and DNA extraction

Bacterial strains used in this study were obtained from collections of Shizuoka University Plant Pathology Laboratory (SUPP) or the National Institute of Agrobiological Science Genebank of Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan (Table 1). The isolates were preserved in a skim milk solution [5 g skim milk (Difco), 0.75 g Na glutamate, 50 mL distilled water] and stored at −20 °C. When required, they were cultured on potato peptone glucose agar (200 g potato, 5 g peptone, 5 g glucose, 3 g Na_2HPO_4·$2H_2O$, 3 g NaCl, 0.5 g KH_2PO_4, 15 g agar, 1000 mL distilled water) (Nishiyama 1978) and incubated at 28 °C for 24 h. Five non-Japanese *Dickeya* strains (SR strains), which had been preserved as genomic DNA were also included, i.e., SR90 (corn), SR120 (corn), SR149 (sugar cane), SR171 (corn) and SR261 (corn). They were previously identified as *E. chrysanthemi* pv. *zeae* by Goto (1979). For PCR restriction fragment length polymorphism (RFLP) analyses, 74 other strains of *E. chrysanthemi* in MAFF collection were used (Supplementary Table 1).

For the extraction of genomic DNA, the strains were cultured in 5 mL yeast peptone (YP) medium (5 g yeast extract, 10 g peptone in 1000 mL distilled water, pH 6.8) and incubated at 27 °C for 24 h. Genomic DNA was extracted from the culture using the cetyltrimethylammonium bromide method described by Ausubel et al. (1987) and used at ~1 μg/μL for molecular investigations.

DNA sequence analysis of *recA*, *dnaX, rpoD and gyrB* and 16S rDNA

PCR amplification

PCR amplification was performed with 100 μL total volume (*recA*, *dnaX*, *rpoD*, *gyrB* or 16S rDNA) containing 10 μL of each primer (5 μM in concentration), 10 μL of 10× Ex *Taq* Buffer (TaKaRa Bio), 8 μL dNTP Mix (TaKaRa Bio), 0.4 μL of 250 U Ex *Taq* (TaKaRa Bio), together with 2 μL TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0), 8 μL DNA template and 51.6 μL sterile distilled water for 16S rDNA, or with 1 μL DNA template and 60.6 μL sterile distilled water for *recA*, *dnaX, rpoD* and *gyrB*. Primers and PCR conditions are detailed in Table 2.

Cloning and DNA sequencing of recA, dnaX, rpoD and gyrB

A PCR product of 16S rDNA was purified using the polyethylene glycol precipitation method of Sakamoto et al. (2004). The purified 16S rDNAs were sent to the BEX Co. (Tokyo, Japan) for sequencing. PCR product of *recA*, *dnaX*, *rpoD* and *gyrB* were separated by electrophoresis in 1 % (w/v) agarose gels with 1× Tris–acetic acid-EDTA (TAE) buffer (pH 8.0) at 100 V and visualized using UV light after staining with ethidium bromide (1 μg/mL). The products were purified by Ready-to-Use system Nucleospin (Macherey-Nagel GmbH & Co. KG, Germany). Then the purified DNAs were cloned into pGEM-T easy vector (Promega) and used to transform *Escherichia coli* DH5α cells according to the manufacturer's instructions. Plasmids of *E. coli* containing *recA*, *dnaX*, *rpoD* and *gyrB* were extracted using a mini preparation and boiling method described by Ausubel et al. (1987). The plasmids were sequenced by the BEX Co.

Phylogenetic analysis

Phylogenetic analyses and construction of phylogenetic trees based on the DNA sequences of *recA*, *dnaX*, *rpoD*, *gyrB* and 16S rDNA were performed by neighbor-joining method (Jukes–Cantor model) using MEGA version 4 (Tamura et al. 2007). Sequence data for *Dickeya* reference strains (Supplementary Table 4) were also included in these analyses.

PCR genomic fingerprinting

The PCRs were run using a DNA thermal cycler (2720 Thermal Cycler, Applied Biosystem) in a total volume of 10 μL containing 1 μL DNA template, 2 μL of 5× Gitschier buffer [83 mM $(NH_4)_2SO_4$, 33.5 mM Tris–HCl pH 8.8, 33.5 mM $MgCl_2$, 33.5 mM EDTA pH 8.8, 150 mM β-Mercaptoethanol, 800 μg/mL bovine serum albumin (BSA; Roche Diagnostics GmbH, Germany)], 0.16 μL of 100× solution of BSA (New England Biolabs, MA, USA), 1 μL dimethylsulfoxide (DMSO), 0.8 μL dNTP Mix

Table 1 Bacterial strains used in this study

Strain	Host	Origin	Year isolated	Identity
SUPP 420 (EKUN)	*Clivia miniata* (bush lily)	Okinawa	1985	Putative new species 1
SUPP 1399 (Cat E)	*Cattleya* sp. (cattleya)	Tochigi	1990	Putative new species 1
SUPP 2739 (ITO 494)	*Cattleya* sp. (cattleya)	Tochigi	1990	Putative new species 1
SUPP 1158 (ECALT)	*Calanthe* sp. (calanthe)	Chiba	1989	*D. zeae*
SUPP 877	*Daucus carota* (carrot)	Shizuoka	1987	*D. dadantii*
SUPP 215	*Dianthus caryophyllus* (carnation)	Shizuoka	1983	*D. dianthicola*
SUPP 2525	*Dianthus caryophyllus* (carnation)	Aichi	2006	*D. dianthicola*
MAFF 311098	*Zea mays* (corn)	Hokkaido	1990	*D. zeae*
SUPP 27 (Corn 801)	*Zea mays* (corn)	Yamagata	1980	*D. zeae*
MAFF 311151	*Cichorium intybus* (chicory)	Saitama	1990	*D. chrysanthemi*
MAFF 311043	*Chrysanthemum* sp. (chrysanthemum)	Hokkaido	1992	*D. chrysanthemi*
SUPP 20 (Chr E 8301)	*Chrysanthemum* sp. (chrysanthemum)	Shizuoka	1983	*D. chrysanthemi*
SUPP 1844	*Chrysanthemum* sp. (chrysanthemum)	Shizuoka	1998	*D. chrysanthemi*
SUPP 1352 (HJ 9)	*Dracaena* sp. (dracaena)	Tokyo	1990	Putative new species 1
MAFF 302132	*Solanum melongena* (eggplant)	Nagasaki	1989	*D. chrysanthemi*
MAFF 301767	*Solanum melongena* (eggplant)	Fukuoka	1983	*D. dadantii*
SUPP 1539 (IrisE 9201)	*Iris* sp. (iris)	Shizuoka	1992	Putative new species 1
MAFF 311149	*Kalanchoe* sp. (kalanchoe)	Saitama	1996	*D. dianthicola*
SUPP 1152 (Onc 891)	*Oncidium* sp. (oncidium)	Chiba	1989	Putative new species 1
SUPP 2738 (ITO 508)	*Oncidium* sp. (oncidium)	Tochigi	1990	Putative new species 1
SUPP 2200 (Ecb-10)	*Prunus persica* (peach)	Yamanashi	2001	*D. dadantii*
SUPP 1034 (PhaE 8801)	*Phalaenopsis* sp. (phalaenopsis)	Shizuoka	1988	Putative new species 1
SUPP 2735 (ITO 216)	*Phalaenopsis* sp. (phalaenopsis)	Shizuoka	1988	Putative new species 1
SUPP 2736 (ITO 356)	*Phalaenopsis* sp. (phalaenopsis)	Shizuoka	1988	Putative new species 1
SUPP 2737 (ITO 437)	*Phalaenopsis* sp. (phalaenopsis)	Tochigi	1989	Putative new species 1
MAFF 311041	*Solanum tuberosum* (potato)	Hokkaido	1992	*D. dianthicola*
MAFF 311042	*Solanum tuberosum* (potato)	Hokkaido	1993	*D. dianthicola*
MAFF 301677	*Solanum tuberosum* (potato)	Niigata	1977	Putative new species 2
SUPP 2565 (K03)	*Solanum tuberosum* (potato)	Shizuoka	2007	*D. dianthicola*
MAFF 106502	*Oryza sativa* (rice)	Mie	1984	*D. zeae*
SUPP 739 (R 8)	*Oryza sativa* (rice)	Shizuoka	1977	*D. zeae*
SUPP 410	*Setaria italica* (setaria)	Tochigi	1985	*D. zeae*
SUPP 2162	*Fragaria* × *ananassa* (strawberry)	Ehime	2001	*D. dadantii*
MAFF 106634	*Ipomoea batatas* (sweet potato)	Miyazaki	1996	*D. dadantii*
MAFF 311172	*Colocasia esculenta* (taro)	Okinawa	1998	Putative new species 1
MAFF 311171	*Colocasia esculenta* (taro)	Okinawa	1998	Putative new species 1
SUPP 2586 (SF-1)	*Colocasia esculenta* (taro)	Yamanashi	2004	Putative new species 1
SUPP 1164 (VND 1)	*Vanda* sp. (vanda)	Chiba	1989	Putative new species 1
SUPP 40 (ALE 8292p)	*Allium fistulosum* (Welsh onion)	Saitama	1982	Putative new species 1
SUPP 2451	*Allium fistulosum* (Welsh onion)	Shizuoka	2004	Putative new species 1
MAFF 302984	*Smallanthus sonchifolius* (yacon)	Kagawa	1992	*D. dianthicola*

Strains: *SUPP* Shizuoka University Plant Pathology, *MAFF* Ministry of Agriculture, Forestry and Fisheries. Names in parentheses are the original strain names

(TaKaRa Bio, Japan), 0.05 μL primer(s) for BOX–PCR (50 μM BOX A1R), ERIC–PCR (50 μM ERIC 1R and 50 μM ERIC 2) or REP–PCR (100 μM REP 1R and 100 μM REP 2I), 0.1 μL of 250 U expand high fidelity DNA polymerase (Roche Applied Science, Germany) and 5.69 μL sterile distilled water for BOX–PCR and 5.64 μL sterile distilled water for ERIC and REP–PCR. Information on primers and PCR conditions are detailed in

Table 2 Primer sets used in this study

Primer	Sequence (5′–3′)	Length of PCR product (bp)	Designed for	Annealing temperatures °C (time)[a]	Reference
ErecA1	GGTAAAGGGTCTATCATGCG	764	*recA* amplification	45 (1 min)	Wilson et al. (2002)
ErecA2	CCTTCACCATACATAATTTGGA				
dnaXf	TATCAGGTYCTTGCCCGTAAGTGG	535	*dnaX* amplification	57–60 (1 min)	Slawiak et al. (2009)
dnaXr	TCGACATCCARCGCYTTGAGATG				
72F	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGG	843	*rpoD* amplification	55–59 (1 min)	Maeda et al. (2006)
70R2	ATAGAAATAACCAGACGTAAGTTNGTRTAYTTYTTNGCDAT				
UP-1E	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	940	*gyrB* amplification	55–59 (1 min)	Yamamoto et al. (1999)
Apr U	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA				
fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	1500	16S rDNA amplification	58 (1 min)	Weisburg et al. (1991)
rP2	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT				
REP1R	IIIICGICGICATCIGGC		REP–PCR	65 (1 min)	Rademaker et al. (1998)
REP2I	ICGICTTATCIGGCCTAC				
ERIC1R	ATGTAAGCTCCTGGGGATTCAG		ERIC–PCR	55 (1 min)	
ERIC2	AAGTAAGTGACTGGGGTGAGCG				
BOX-A1R	CTACGGCAAGGCGACGCTGACG		BOX–PCR	55 (1 min)	
RS-1	TTCATRCGRATCTGGTTGAT	499	*recA* PCR–RFLP	56 (30 s)	Present study
RS-2	ATCGCTCAATGGATGTTGAAA				
rpo-RS1	GACCCGTGAAGGGGAAATCG	561	*rpoD* PCR–RFLP	64 (30 s)	Present study
rpo-RS2	TTCTTCGGCATTTTGCACAG				
gyr-RS1	TCCGGCGGTYTGCACGGGGT	740	*gyrB* PCR–RFLP	64 (30 s)	Present study
gyr-RS2	AGACGGTCGTTCATCAGCGA				

[a] For *recA*, *dnaX*, *rpoD*, *gyrB* and 16S rDNA amplification, initial denaturation: at 94 °C for 5 min; PCR amplification: 30 cycles with denaturation at 94 °C for 1 min; primer extension: 72 °C for 1 min (*recA*, *dnaX*, *rpoD* and *gyrB*) and 3 min (16S rDNA); final elongation: 72 °C for 7 min (*recA*, *dnaX*, *rpoD* and *gyrB*) or 10 min (16S rDNA). For *recA*, *rpoD* and *gyrB* amplification for PCR RFLP, initial denaturation: 94 °C for 5 min; PCR amplification: 32 cycles (*recA*) and 30 cycles (*rpoD*, *gyrB*) with denaturation at 94 °C for 30 s; primer extension: 72 °C for 30 s; elongation step: 72 °C for 7 min; For repetitive genomic fingerprinting, initial denaturation: 94 °C for 2 min; PCR amplification: 30 cycles denaturation at 94 °C for 3 s, then 92 °C for 30 s, primer extension at 65 °C for 8 min, and final elongation step of 65 °C for 8 min

Table 2. The PCR products were run in 1.5 % agarose gels using 1× TAE buffer (pH 8.0) at 50 V and visualized using UV light after staining with ethidium bromide (1 μg/mL).

PCR–RFLP analysis of *recA*, *rpoD* and *gyrB*

Primer design for recA, rpoD and gyrB

When the aforementioned primers were used for amplification of *recA*, *rpoD* and *gyrB* genes, extra bands that might interfere in further analyses appeared. Therefore, new primers were designed to gain a single-band PCR product to use for RFLP analysis: for *recA*, primers RS-1 and RS-2; *rpoD*, rpo-RS1 and rpo-RS2; *gyrB*, gyr-RS1 and gyr-RS2 (Table 2).

DNA amplification using new primers

DNA was amplified in a total volume of 20 μL containing 1 μL DNA template, 2 μL of each primer (5 μM concentration), 2 μL of 10× Ex *Taq* buffer (TaKaRa Bio), 1.6 μL dNTP Mix (TaKaRa Bio), 0.08 μL of 250 U Ex *Taq* (TaKaRa Bio) and 13.32 μL sterile distilled water. Information on the primers and PCR conditions are detailed in Table 2.

PCR–RFLP of recA, rpoD and gyrB

The amplified PCR product (using the new primers described already) for *recA* was digested with six restriction enzymes (SalI, BglII, PstI, SphI, HaeII and AluI), with three restriction enzymes (HindfI, DdeI and BglII) for *rpoD* and two restriction enzymes (BamHI and DdeI) for

gyrB, selected according to the nucleotide sequence of the respective genes in the Japanese *Dickeya* strains using the program DNASIS-Mac ver. 3 (Hitachi Solutions, Japan). All buffers, BSA and endonucleases used were obtained from New England BioLabs. Conditions for the restriction analyses for the three genes were as follows: *recA*—total volume 10 µL (5 µL PCR product, 1 µL 10× NE buffer 3, 0.5 µL 100× BSA solution, 0.5 µL of restriction enzyme (SphI 10,000 U/mL, AluI 10,000 U/mL, SalI 20,000 U/mL, BglII 10,000 U/mL, PstI 20,000 U/mL and HaeII 20,000 U/mL) and 0.5 µL sterile distilled water; *rpoD*—total volume 10 µL (6 µL of PCR product, 1 µL of 10× NE buffer 2, 0.5 µL of each restriction enzyme (i.e., DdeI 10,000 U/mL, hinf1 10,000 U/mL and BglII 10,000 U/mL), and 1.5 µL sterile distilled water); *gyrB*—total volume of 10 µL (6 µL of PCR product, 1 µL 10× NE buffer 3, 0.5 µL of 100× BSA solution, 0.5 µL of each restriction enzyme (DdeI 10,000 U/mL, BamHI 20,000 U/mL), and 1.5 µL sterile distilled water). Digested *recA* samples were incubated for ~48 h at 37 °C those for *rpoD* and *gyrB* were incubated for ~2 h at 37 °C. Restriction fragments were run in 1.10 agarose gels using 1× TAE buffer (pH 8.0) at 50 V and visualized using UV light after staining with ethidium bromide (1 µg/mL).

Phenotypic properties

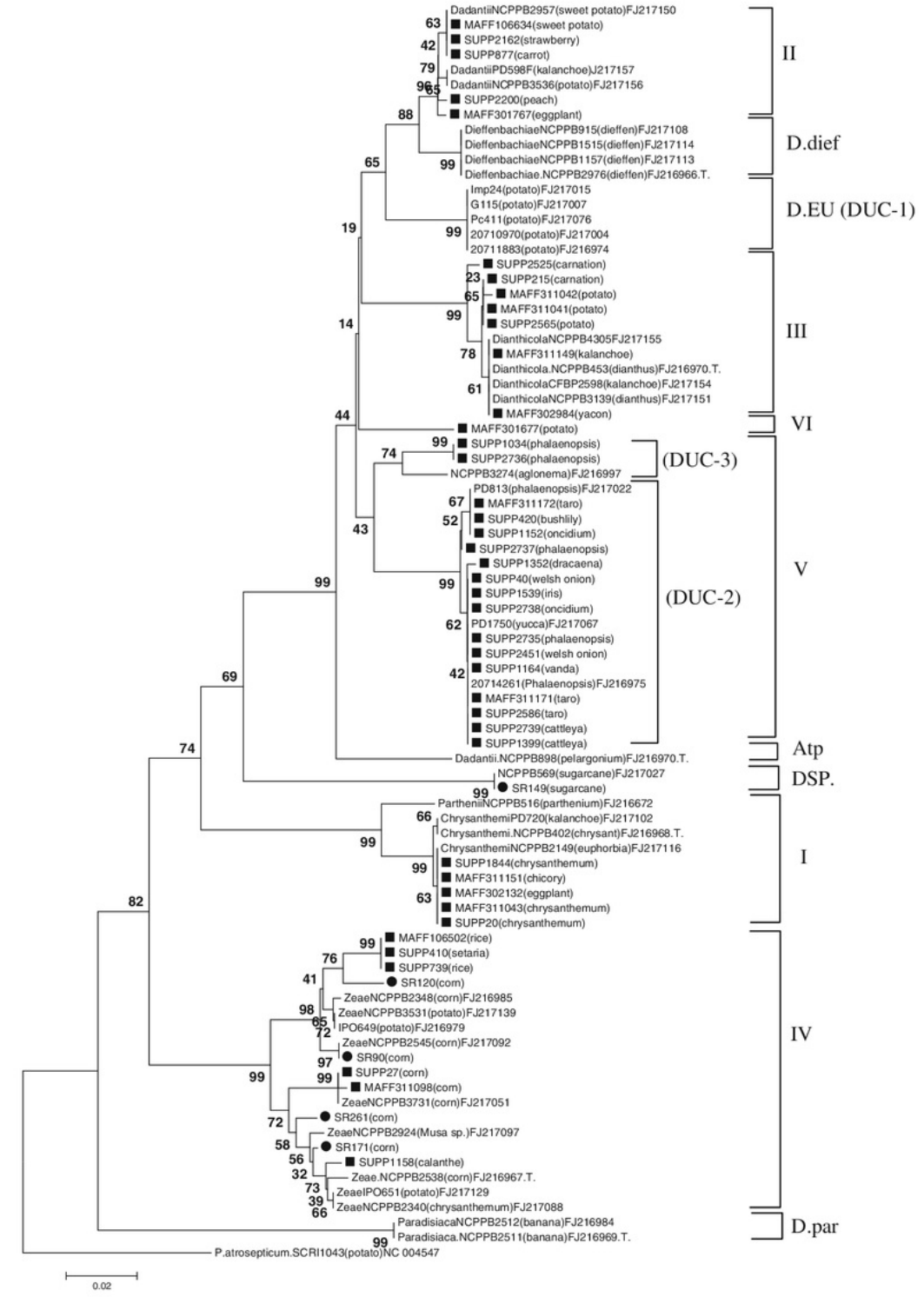
Gram reaction was examined using a nonstaining method with 3 % KOH solution described by Ryu (1940). Fluorescent pigment production was determined on medium B of King et al. (1954). Hugh and Leifson's (1953) medium was used for the oxidation/fermentation (OF) test. Potato soft rot test, oxidase activity, Thornley's arginine dihydrolase, nitrate reduction, gelatin liquefaction, hydrolysis of Tween 80, egg yolk reaction (lecithinase activity), and hydrolysis of casein were performed using the methods described by Lelliot et al. (1966). Moeller's arginine dihydrolase test was performed using decarboxylase base Moeller medium (Difco) with 1 % of L-arginine hydrochloride (Dickey 1979). The methods described by Dye (1968) were used to test for H_2S production, indole production, acetoin production, reducing substances from sucrose, pectate liquefaction, blue pigment production on yeast dextrose chalk agar medium, methyl red test, growth at 5 % NaCl, indole production, phosphatase and catalase tests. Utilization of 40 organic compounds as a sole source of carbon was tested on the modified medium of Ayers et al. (Society of American Bacteriologist 1957), with 0.1 % (w/v) organic compounds incorporated. A positive reaction was assessed when bacterial growth was observed within 21 days at 27 °C. Growth at 39, 40 and 41 °C were tested in YP medium.

Fig. 1 Phylogenetic tree of the *recA* sequence reconstructed using MEGA4 (Tamura et al. 2007) and a neighbour-joining method. The tree was rooted using the sequence of *Pectobacterium atrosepticum* strain SCRI 1043 (GenBank accession NC_004547). The type strains (T) of six known *Dickeya* species were also included. All reference strains were obtained from the study by Parkinson et al. (2009). Group I: *D. chrysanthemi*; group II: *D. dadantii*; group III: *D. dianthicola*; group IV: *D. zeae*; group V: putative new species 1; group VI: putative new species 2; D. dief: *D. dieffenbachiae* (Parkinson et al. 2009); D.par: *D. paradisiaca* (Parkinson et al. 2009); D.EU: Unknown representative *Dickeya* spp. from Europe (Parkinson et al. 2009). *Atp* atypical *Dickeya dadantii* type strain (Parkinson et al. 2009); *DSP* new clade reported by Parkinson et al. (2009). DUC-1, DUC-2, and DUC-3 are the *Dickeya* species-level groups reported by Parkinson et al. (2009)

Results

Phylogenetic analysis of *recA*, *dnaX, rpoD, gyrB* and 16S rDNA sequences

On the basis of *recA* (Fig. 1) sequence analysis, the Japanese *Dickeya* strains separated into six genetic groups. The strains isolated from chrysanthemum (MAFF 311043, SUPP 20 and SUPP 1844), eggplant (MAFF 302132) and chicory (MAFF 311151) were placed together with the type strain of *D. chrysanthemi* (NCPPB 402 acc. FJ216968), named here as group I. The strains isolated from peach (SUPP 2200), eggplant (MAFF 301767), sweet potato (MAFF 106634), strawberry (SUPP 2162) and carrot (SUPP 877) were placed with some of the reference strains of *D. dadantii* (NCPPB 2957 acc. FJ217150; PD 598 acc. 217157; NCPPB 3536 acc. FJ217156), named here as group II. In contrast, the type strain of *D. dadantii* (NCPPB 898 acc. FJ216970) was placed separately from *D. dadantii* group. The strains that were isolated from carnation (SUPP 215 and SUPP 2525), potato (MAFF 311041, MAFF 311042 and SUPP 2565), yacon (MAFF 302984) and *Kalanchoe* (MAFF 311149) were placed with the type strain of *D. dianthicola* (NCPPB 453 acc. FJ216970) and named here as group III. Strains from corn (MAFF 311098 and SUPP 27), rice (MAFF 106502 and SUPP 739), *Setaria* (foxtail millet) (SUP32.10) and *Calanthe* (SUPP 1158) were placed with the type strain of *D. zeae* (NCPPB 2538 acc. FJ216967) and named group IV. Strains from taro (SUPP 2586, MAFF 311171 and MAFF 311172), Welsh onion (SUPP 2451 and SUPP 40), *Phalaenopsis* (SUPP 1034, SUPP 2735, SUPP 2737 and SUPP 2736), *Cattleya* (SUPP 1399 and SUPP 2739), *Oncidium* (SUPP 1152 and SUPP 2738), *Dracaena* (SUPP 1352), *Vanda* (SUPP 1164) and iris (SUPP 1539) were grouped together and called group V, and one strain from potato (MAFF 301677) was called group VI. Groups V and VI could not be allocated to any known *Dickeya* species groups and may constitute two putative new species.


DadantiiNCPPB2957(sweet potato)FJ217150
MAFF106634(sweet potato)
SUPP2162(strawberry)
SUPP877(carrot)
DadantiiPD598F(kalanchoe)J217157
DadantiiNCPPB3536(potato)FJ217156
SUPP2200(peach)
MAFF301767(eggplant)
DieffenbachiaeNCPPB915(dieffen)FJ217108
DieffenbachiaeNCPPB1515(dieffen)FJ217114
DieffenbachiaeNCPPB1157(dieffen)FJ217113
Diffenbachiae.NCPPB2976(dieffen)FJ216966.T.
Imp24(potato)FJ217015
G115(potato)FJ217007
Pc411(potato)FJ217076
20710970(potato)FJ217004
20711883(potato)FJ216974
SUPP2525(carnation)
SUPP215(carnation)
MAFF311042(potato)
MAFF311041(potato)
SUPP2565(potato)
DianthicolaNCPPB4305FJ217155
MAFF311149(kalanchoe)
Dianthicola.NCPPB453(dianthus)FJ216970.T.
DianthicolaCFBP2598(kalanchoe)FJ217154
DianthicolaNCPPB3139(dianthus)FJ217151
MAFF302984(yacon)
MAFF301677(potato)
SUPP1034(phalaenopsis)
SUPP2736(phalaenopsis)
NCPPB3274(aglonema)FJ216997
PD813(phalaenopsis)FJ217022
MAFF311172(taro)
SUPP420(bushlily)
SUPP1152(oncidium)
SUPP2737(phalaenopsis)
SUPP1352(dracaena)
SUPP40(welsh onion)
SUPP1539(iris)
SUPP2738(oncidium)
PD1750(yucca)FJ217067
SUPP2735(phalaenopsis)
SUPP2451(welsh onion)
SUPP1164(vanda)
20714261(Phalaenopsis)FJ216975
MAFF311171(taro)
SUPP2586(taro)
SUPP2739(cattleya)
SUPP1399(cattleya)
Dadantii.NCPPB898(pelargonium)FJ216970.T.
NCPPB569(sugarcane)FJ217027
SR149(sugarcane)
ParthenniNCPPB516(parthenium)FJ216672
ChrysanthemiPD720(kalanchoe)FJ217102
Chrysanthemi.NCPPB402(chrysant)FJ216968.T.
ChrysanthemiNCPPB2149(euphorbia)FJ217116
SUPP1844(chrysanthemum)
MAFF311151(chicory)
MAFF302132(eggplant)
MAFF311043(chrysanthemum)
SUPP20(chrysanthemum)
MAFF106502(rice)
SUPP410(setaria)
SUPP739(rice)
SR120(com)
ZeaeNCPPB2348(corn)FJ216985
ZeaeNCPPB3531(potato)FJ217139
IPO649(potato)FJ216979
ZeaeNCPPB2545(corn)FJ217092
SR90(com)
SUPP27(corn)
MAFF311098(com)
ZeaeNCPPB3731(corn)FJ217051
SR261(com)
ZeaeNCPPB2924(Musa sp.)FJ217097
SR171(corn)
SUPP1158(calanthe)
Zeae.NCPPB2538(corn)FJ216967.T.
ZeaeIPO651(potato)FJ217129
ZeaeNCPPB2340(chrysanthemum)FJ217088
ParadisiacaNCPPB2512(banana)FJ216984
Paradisiaca.NCPPB2511(banana)FJ216969.T.
P.atrosepticum.SCRI1043(potato)NC 004547
II
D.dief
D.EU (DUC-1)
III
VI
(DUC-3)
(DUC-2)
V
Atp
DSP.
I
IV
D.par
0.02
Dickeya spp. used in this study
Dickeya spp. from countries other than Japan (Goto 1979)

The *dnaX* sequence analysis (Supplementary Figure 1) gave the same results as *recA* did (Fig. 1). All the strains within group I were placed with the type strain of *D. chrysanthemi* (IPO 2118 acc. GQ904750), those of group II were placed with *D. dadantii* (IPO 2120 acc. GQ904753), and group III strains were placed with *D. dianthicola* (IPO 2114 acc. GQ904747). Group IV strains were placed with reference strains of *D. zeae* (IPO 649 acc. GQ904770; IPO 450 acc. GQ904771; IPO 651 acc. GQ905772) except for the type strain of *D. zeae* (IPO 2131 acc. GQ904764), which was placed with the other two reference strains of *D. zeae* (IPO 2132 acc. GQ904765; IPO 2133 acc. GQ904766). On the other hand, the strains within group V and in VI could not be assigned to any known *Dickeya* species groups and may constitute two putative new species (Supplementary Figure 1). Four SR strains (SR 90, 120, 171 and 261) were placed in the same group with *D. zeae*, while SR149 formed an independent branch, which may become another putative new species-level clade (Supplementary Figure 1).

The results of *rpoD* (Supplementary Figure 2) and *gyrB* (Supplementary Figure 3) sequence analysis corresponded with those for *recA* and *dnaX*. In the case of *gyrB*, one exception was found: MAFF 301677 isolated from potato (group VI of the *rpoD*, *recA* and *dnaX* sequence analyses) was placed in group III (*D. dianthicola*) (Supplementary Figure 3).

On the basis of 16S rDNA, groups I–VI showed the same results as the other gene analyses; again, strains that were placed in group V or VI were also placed in clusters independent of those with the other known *Dickeya* species. However, by this method, two group III strains, MAFF 311149 (isolated from *Kalanchoe*) and SUPP 2525 (isolated from carnation), were placed in a group independent of groups I–VI and named here as group III* (Supplementary Figure 4).

All nucleotide sequence data are available in the DDBJ/EMBL/GenBank database (accession numbers are in Supplementary Table 5).

PCR genomic fingerprinting

The bands produced by REP–PCR, ERIC–PCR and BOX–PCR revealed distinctive patterns for the Japanese *Dickeya* strains according to their respective genetic groups (I–VI, Table 3). However, they could not be clearly distinguished with the naked eye. For example, MAFF 301677 (isolated from potato), which was placed in group VI in the *recA*, *dnaX*, *rpoD* and 16S rDNA analyses, had the same pattern as the group V strains in REP, ERIC and BOX–PCR (Supplementary Figure 8).

PCR–RFLP of *recA, rpoD* and *gyrB* gene fragment analysis

The new grouping method using PCR–RFLP analysis of *recA*, *rpoD* and *gyrB* genes was developed based on the DNA sequences of those genes. Here, we showed that on the basis of PCR–RFLP of *recA*, *rpoD* and *gyrB* genes, each of the 41 Japanese *Dickeya* strains tested could be assigned to either one or the other genetic groups based on the DNA sequences (Table 3).

The PCR–RFLP of *recA* gene, digested with SalI, BglII, PstI, SphI, HaeII and AluI (Table 4) revealed that group I (*D. chrysanthemi*) produced bands at 135, 166 and 198 bp (rec.I); group II (*D. dadantii*) produced bands at 135, 143 and 221 bp (rec.II); and group III (*D. dianthicola*) produced bands at 84, 194 and 221 bp (rec.III). The strains within group IV (*D. zeae*) produced two kinds of band patterns, i.e., 63, 135 and 301 bp (rec.IV.1); and 63, 103, 135 and 198 bp (rec.IV.2). The strains belonging to group V (putative new species 1) produced three kinds of band patterns, i.e., 8, 213 and 278 bp (rec.V.1); 8, 135, 143 and 213 bp (rec.V.2); and 135, 151 and 213 bp (rec.V.3). The strain in group VI (putative new species 2) produced a distinct pattern of bands at 221 and 278 bp (rec.VI). The gel image of *recA* PCR–RFLP pattern is shown in Fig. 2.

This method was also applied to five SR (*E. chrysanthemi* pv. *zeae*) strains. Strain SR90, 120, 171 and 261 yielded the same bands as the Japanese *Dickeya zeae* strains [rec.IV.1 (SR171, SR261); rec.IV.2 (SR90, SR120)]. On the other hand, SR149 yielded different bands ($\sim$190 and 300 bp) than the Japanese strains of *D. zeae* or the other *Dickeya* spp. did (Supplementary Figure 5).

The PCR–RFLP of *rpoD* gene digested with HinfI, DdeI and BglII (Table 5) showed that group I (*D. chrysanthemi*) produced bands at 23, 33, 54, 151 and 300 bp (rpo.I); group II (*D. dadantii*) produced bands at 33, 151 and 377 bp (rpo.II); and group III (*D. dianthicola*) produced bands at 33, 151, 168 and 209 bp (rpo.III). The strains members of group IV (*D. zeae*) produced three kinds of band patterns, i.e., 33, 141 and 387 bp (rpo.IV.1); 33, 54, 61, 84, 87 and 242 bp (rpo.IV.2); and 33, 61, 84, 141 and 242 bp (rpo.IV.3). The strains within group V (putative new species 1) produced two kinds of band patterns, i.e., 33, 242 and 286 bp (rpo.V.1); and 33, 91, 151 and 286 bp (rpo.V.2). The strains in group VI (putative new species 2) produced bands at 33, 141, 151 and 236 bp (rpo.VI). The gel image of *rpoD* PCR–RFLP pattern is shown in Fig. 3. In the case of SR strains, SR 120, 171 and 261 produced the same bands as Japanese *Dickeya zeae* strains (rpo.IV.2), but SR 90 and SR 149 produced different bands (approximately 60, 80 and 380 bp for SR 90 and 150 and 200 bp for SR 149) than the

Table 3 Comparison of taxa generated by the analyses of PCR genomic fingerprinting, MLSA, biovar, phenon and PCR–RFLP of *gyrB*, *rpoD* and *recA*

Strain	Host	PCR genomic fingerprinting group			DNA sequence analysis group					PCR–RFLP group			Biovar	Phenon	Identity
		ERIC	REP	BOX	16SrDNA	*gyrB*	*rpoD*	*recA*	*dnaX*	*gyrB*	*rpoD*	*recA*			
SUPP1844	Chrysanthemum	I	I	I	I	I	I	I	I	gyr.I	rpo.I	rec.I	5	4	*D. chrysanthemi*
SUPP20	Chrysanthemum	I	I	I	I	I	I	I	I	gyr.I	rpo.I	rec.I	5	4	*D. chrysanthemi*
MAFF 311043	Chrysanthemum	I	I	I	I	I	I	I	I	gyr.I	rpo.I	rec.I	5	4	*D. chrysanthemi*
MAFF 311151	Chicory	I	I	I	I	I	I	I	I	gyr.I	rpo.I	rec.I	5	4	*D. chrysanthemi*
MAFF 302132	Eggplant	I	I	I	I	I	I	I	I	gyr.I	rpo.I	rec.I	5	4	*D. chrysanthemi*
MAFF 301767	Eggplant	II	II	II	II	II	II	II	II	gyr.II.2	rpo.II	rec.II	3	1	*D. dadantii*
SUPP2200	Peach	II	II	II	II	II	II	II	II	gyr.II.1	rpo.II	rec.II	3	1	*D. dadantii*
SUPP877	Carrot	II	II	II	II	II	II	II	II	gyr.II.1	rpo.II	rec.II	3	1	*D. dadantii*
SUPP2162	Strawberry	II	II	II	II	II	II	II	II	gyr.II.1	rpo.II	rec.II	3	1	*D. dadantii*
MAFF 106634	Sweet potato	II	II	II	II	II	II	II	II	gyr.II.1	rpo.II	rec.II	3	1	*D. dadantii*
MAFF 302984	Yacon	III	III	III	III	III	III	III	III	gyr.III.1/V.2	rpo.III	rec.III	1	5	*D. dianthicola*
SUPP2525	Carnation	III	III	III	III*	III	III	III	III	gyr.III.2	rpo.III	rec.III	1*	5	*D. dianthicola*
MAFF 311149	*Kalanchoe* sp.	III	III	III	III*	III	III	III	III	gyr.III.2	rpo.III	rec.III	9	5	*D. dianthicola*
SUPP215	Carnation	III	III	III	III	III	III	III	III	gyr.III.2	rpo.III	rec.III	1	5	*D. dianthicola*
SUPP2565	Potato	III	III	III	III	III	III	III	III	gyr.III.2	rpo.III	rec.III	1	5	*D. dianthicola*
MAFF 311041	Potato	III	III	III	III	III	III	III	III	gyr.III.2	rpo.III	rec.III	1	5	*D. dianthicola*
MAFF 311042	Potato	III	III	III	III	III	III	III	III	gyr.III.1/V.2	rpo.III	rec.III	1	5	*D. dianthicola*
MAFF 106502	Rice	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.2	rpo.IV.3	rec.IV.2	3	1	*D. zeae*
SUPP739	Rice	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.2	rpo.IV.3	rec.IV.2	3	1	*D. zeae*
SUPP 410	*Setaria italica*	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.2	rpo.IV.2	rec.IV.2	3	1	*D. zeae*
SUPP1158	*Calanthe* sp.	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.1	rpo.IV.2	rec.IV.1	3	1	*D. zeae*
MAFF 311098	Corn	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.1	rpo.IV.1	rec.IV.1	8	1	*D. zeae*
SUPP27	Corn	IV	IV	IV	IV	IV	IV	IV	IV	gyr.IV.1	rpo.IV.1	rec.IV.1	8	1	*D. zeae*
SR 90[s1]	Corn	NT	NT	NT	NT	NT	NT	IV	IV	gyrIV.1	ukn	recIV.2	NT	NT	*D. zeae*
SR 120[s2]	Corn	NT	NT	NT	NT	NT	NT	IV	IV	gyrIV.1	rpoIV.2	recIV.2	NT	NT	*D. zeae*
SR 171[s3]	Corn	NT	NT	NT	NT	NT	NT	IV	IV	gyrIV.1	rpoIV.2	recIV.1	NT	NT	*D. zeae*
SR 261[s4]	Corn	NT	NT	NT	NT	NT	NT	IV	IV	gyrIV.3	rpoIV.2	recIV.1	NT	NT	*D. zeae*
SR 149[s5]	Sugarcane	NT	NT	NT	NT	NT	NT	DSP	DSP	ukn	Ukn	Ukn	NT	NT	Another putative new species[a]
SUPP2451	Welsh onion	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.1	rec.V.1	3	1*	Putative new species 1
SUPP40	Welsh onion	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.1	3	1*	Putative new species 1
SUPP1034	*Phalaenopsis* sp.	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.1	rec.V.3	3	1*	Putative new species 1
SUPP2736	*Phalaenopsis* sp.	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.1	rec.V.3	3	1	Putative new species 1
SUPP2737	*Phalaenopsis* sp.	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.2	3	1*	Putative new species 1
SUPP2735	*Phalaenopsis* sp.	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.1	rec.V.1	3	1*	Putative new species 1

Table 3 continued

Strain	Host	PCR genomic fingerprinting group			DNA sequence analysis group					PCR–RFLP group			Biovar	Phenon	Identity
		ERIC	REP	BOX	16SrDNA	*gyrB*	*rpoD*	*recA*	*dnaX*	*gyrB*	*rpoD*	*recA*			
SUPP2586	Taro	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.1	3	1*	Putative new species 1
MAFF 311171	Taro	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.1	rec.V.2	3	1*	Putative new species 1
MAFF 311172	Taro	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.1	rec.V.2	3	1*	Putative new species 1
SUPP420	Bush lily	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.2	3	I*	Putative new species 1
SUPP1152	*Oncidium* sp.	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.2	rec.V.2	3	1*	Putative new species 1
SUPP2738	*Oncidium* sp.	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.1	rec.V.1	3	1*	Putative new species 1
SUPP1164	*Vanda* sp.	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.1	3	1*	Putative new species 1
SUPP1352	*Dracaena* sp	V	V	V	V	V	V	V	V	gyrV.1	rpo.V.1	rec.V.1	3	1*	Putative new species 1
SUPP1399	*Cattleya* sp	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.1	rec.V.1	3	1	Putative new species 1
SUPP2739	*Cattleya* sp.	V	V	V	V	V	V	V	V	gyr.V.2/III.1	rpo.V.1	rec.V.1	3	1*	Putative new species 1
SUPP1539	Iris	V	V	V	V	V	V	V	V	gyr.V.1	rpo.V.2	rec.V.1	3	1	Putative new species 1
MAFF 301677	Potato	V	V	V	VI	III	VI	VI	VI	gyr.III.2/V.1	rpo.VI	rec.VI	3	1*	Putative new species 2

SUPP Shizuoka University Plant Pathology, *s1* isolated in India, *s2* isolated in Hawaii, *s3* isolated in Colombia, *s4* isolated in Costa Rica, *s5* isolated in Australia, *NT* not tested, *Ukn* unknown. [a]different clade from putative new species 1 and 2; DSP: SR 149 gave unassigned RFLP pattern from the other Japanese *Dickeya* species in *gyrB*, *rpoD* and *recA* RFLP method. III*: Strains were placed in a group separate from *D. dianthicola*, but the BLAST result indicated that strains are close to *D. dianthicola*. Biovar 1* (described in this study) was close to biovar 1, but was negative for utilization of D-tartrate. Phenon 1* (described in present study) was close to phenon 1, but was negative for utilization of *cis*-aconitate

Table 4 Size of bands produced with *recA* PCR–RFLP after restriction enzyme digestion according to RFLP group and *Dickeya* group

Data	Restriction enzyme	RFLP group								
		rec.I	rec.II	rec.III	rec.IV.1	rec.IV.2	rec.V.1	rec.V.2	rec.V.3	rec.VI
Positions of digestion site	SalI	333	0	0	0	333	0	0	0	0
	BglII	0	0	0	0	0	286	286	286	0
	PstI	135	135	0	135	135	0	135	135	0
	SphI	0	0	194	0	0	0	0	0	0
	HaeII	0	278	278	0	0	278	278	0	278
	AluI	0	0	0	436	436	0	0	0	0
Band size produced (bp)		135	135	84	63	63	8	8	135	221
		166	143	194	135	103	213	135	151	278
		198	221	221	301	135	278	143	213	
						198		213		
Dickeya spp. (group)		*chrysanthemi* (I)	*dadantii* (II)	*dianthicola* (III)	*zeae* (IV)		putative new species 1 (V)			putative new species 2 (VI)

PCR product was 499 bp long. Products after digestion were assessed on the basis of sequence results of all 41 Japanese *Dickeya* strains used in the present study. Bands less than 50 bp cannot be visualized after gel electrophoresis

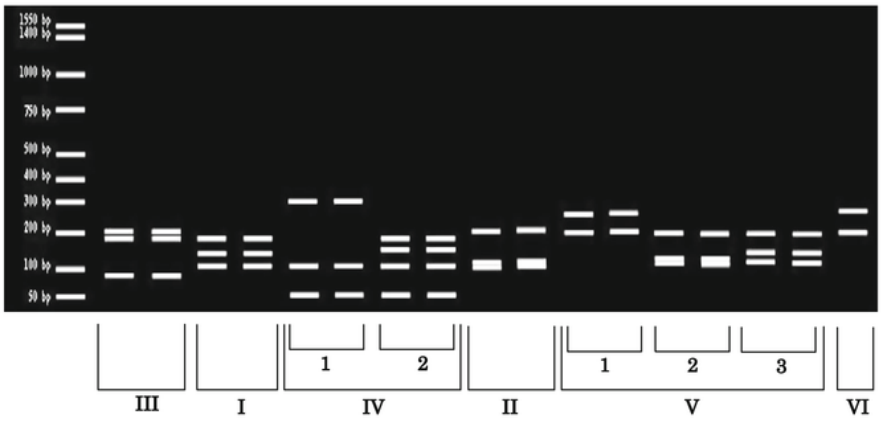


Fig. 2 Virtual gel image of *recA* PCR–RFLP pattern. The marker was designed based on the partial band pattern of Hi-LO DNA Marker (Bionexus, Oakland, CA). Combination of six restriction enzymes (SalI, BglII, PstI, SphI, HaeII and AluI) were used. Group I: *D. chrysanthemi*; group II: *D. dadantii*; group III: *D. dianthicola*; group IV: *D. zeae*; group V: putative new species 1; group V: putative new species 2. No. 1, 2, 3: subgroups of band patterns produced by each RFLP group. Bands less than 50 bp are not large enough to be visible

Japanese *D. zeae* strains and the other Japanese *Dickeya* species (Supplementary Figure 6).

In the case of PCR–RFLP of *gyrB* gene digested with BamHI and DdeI (Table 6), the strains in group I (*D. chrysanthemi*) produced bands at 230 and 510 bp (gyr.I). Strain members of group II (*D. dadantii*) produced two kinds of band patterns, i.e., 127 and 613 bp (gyr.II.1); and 69, 127 and 544 bp (gyr.II.2). Strains within group III (*D. dianthicola*) also produced two kinds of band patterns, i.e., 44 and 696 bp (gyr.III.1) and 86 and 654 bp (gyr.III.2). Strains in group IV (*D. zeae*) produced two kinds of band patterns, i.e., 86, 103, 127 and 424 bp (gyr.IV.1) and 47, 80, 103 and 510 bp (gyr.IV.2) The strains in group V (putative new species 1) produced two kinds of band patterns, i.e., 86, 127 and 527 bp (gyr.V.1) and 86 and 654 bp (gyr.V.2). Furthermore, with this method, group VI (putative new species 2) showed the same result as group III (*D. dianthicola*), which produced bands at 44 and 696 bp (gyr.III.1). In addition, the strains within the RFLP group of gyr.III.1 produced the same bands as the strains in RFLP group V.2 (gyr.III.1 = gyr.V.2), meaning that the *gyrB* RFLP method cannot be used as the final or the only method to identify the *Dickeya* spp. The gel image of the *gyrB* PCR–RFLP pattern can be seen in Fig. 4. In the case of SR strains, SR 90, 120 and SR 171 produced the same

Table 5 Size of bands produced with *rpoD* PCR–RFLP after restriction enzyme digestion according to RFLP group and *Dickeya* group

Data	Restriction enzyme	RFLP group								
		rpo.I	rpo.II	rpo.III	rpo.IV.1	rpo.IV.2	rpo.IV.3	rpo.V.1	rpo.V.2	rpo.VI
Positions of digestion site	HinfI	33	33	33	33	33	33	33	33	33
				352		275	275	275	275	
	DdeI	207			420	359	359	0	0	420
		507				420	420			
						507				
	BglII	184	184	184	0	0	0	0	184	184
Bands produced (bp)		23	33	33	33	33	33	33	33	33
		33	151	151	141	54	61	242	91	141
		54	377	168	387	61	84	286	151	151
		151		209		84	141		286	236
		300				87	242			
						242				
Dickeya spp. (group)		*chrysanthemi* (I)	*dadantii* (II)	*dianthicola* (III)	*zeae* (IV)			putative new species 1 (V)		putative new species 2 (VI)

PCR product was 561 bp long. Products after digestion were assessed on the basis of sequence results of all 41 Japanese *Dickeya* strains used in the present study. Bands less than 50 bp cannot be visualized after gel electrophoresis

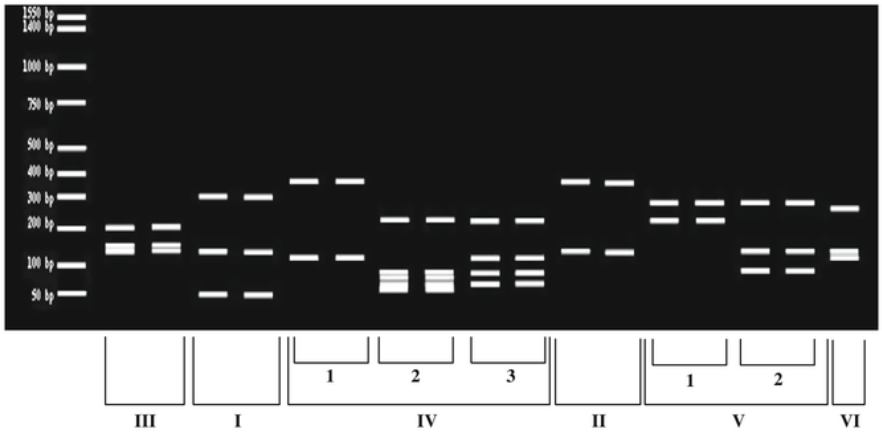


Fig. 3 Virtual gel image of *rpoD* PCR–RFLP pattern. The marker was designed based on the partial band pattern of Hi-LO DNA Marker (Bionexus). Combination of three restriction enzymes (Hinf1, DdeI and BglII) were used. Group I: *D. chrysanthemi*; group II: *D. dadantii*; group III: *D. dianthicola*; group IV: *D. zeae*; group V: putative new species 1; group VI: putative new species 2. No. 1, 2, 3: subgroups of band patterns produced by each RFLP group. Bands less than 50 bp are not large enough to be visible

bands as the Japanese *Dickeya zeae* strains (gyr.IV.1), while SR 261 and 149 produced different bands (approximately 100, 200 and 400 bp for SR 261 and 100 and 500 bp for SR 149) than the Japanese *D. zeae* strains or the other Japanese *Dickeya* spp. (Supplementary Figure 7).

Using a combination of those three PCR–RFLP methods, we can easily and rapidly identify 74 additional *Dickeya* (=*E. chrysanthemi*) strains from the MAFF collection (Supplementary Table 1).

Phenotypic identification

Based on the results of biochemical tests, the 41 Japanese *Dickeya* strains were grouped into three phena (phenon 1, 4, 5) including phenon 1*, or five biovars (biovar 1, 3, 5, 8, 9) including biovar 1* (Table 3, Supplementary Table 2). Phenon 1* was close to phenon 1, but was negative for utilization of *cis*-aconitate (Table 3, Supplementary Table 3). Biovar 1* (consisting of one of the carnation isolates, SUPP 2525) was close to biovar 1, but was

Table 6 Size of bands produced by *gyrB* PCR–RFLP after restriction enzyme digestion according to RFLP group and *Dickeya* group

Data	Restriction enzyme	RFLP group									
		gyr.I	gyr.II.1	gyr.II.2	gyr.III.1	gyr.III.2		gyr.IV.1	gyr.IV.2	gyr.V.1	gyr.V.2
Position of restriction enzyme cut site	*Bam*HI	0	0	0	654	0	0	654	0	654	654
	*Dde*I	230	127	127		696	696	127	47	127	
				671				230	127		
									230		
Bands produced (bp)		230	127	69	86	44	44	86	47	86	86
		510	613	127	654	696	696	103	80	127	654
				544				127	103	527	
								424	510		
Dickeya spp. (group)		*chrysanthemi* (I)	*dadantii* (II)		*dianthicola* (III)		Putative new species 2; MAFF301677 (VI)	*zeae* (IV)		Putative new species 1 (V)	

PCR product was 740 bp long. Products after digestion were assessed on the basis of sequence results of all 41 Japanese *Dickeya* strains used in the present study. Bands less than 50 bp cannot be visualized after gel electrophoresis

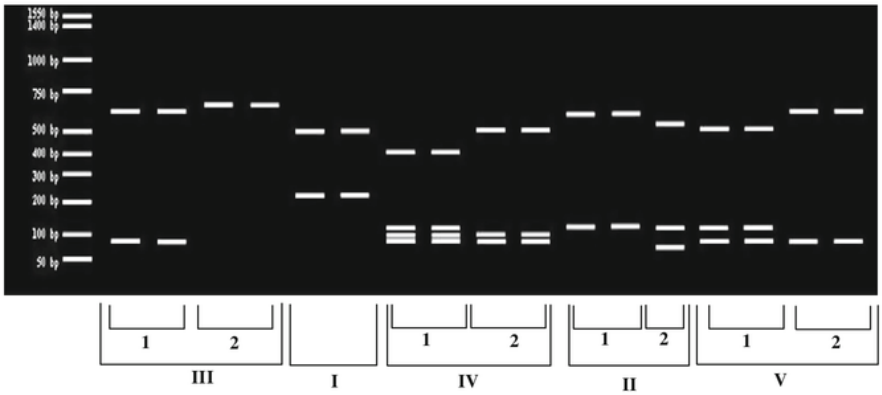


Fig. 4 Virtual gel image of *gyrB* PCR–RFLP pattern. The marker was designed based on the partial band pattern of Hi-LO DNA Marker (Bionexus). Combination of two restriction enzymes (BamHI and DdeI) were used in this method. Using this method, Japanese *Dickeya* strains were clustered into five groups. Group I: *D. chrysanthemi;* group II: *D. dadantii*; group III: *D. dianthicola*; group IV: *D. zeae*; group V: putative new species 1. By this method, strain MAFF301677 produced the same pattern as strains of group III. No. 1, 2: subgroups of band patterns produced by each RFLP group. Bands less than 50 bp are not large enough to be visible

negative for utilization of D-tartrate (Supplementary Table 2). Group I (*D. chrysanthemi*) corresponded to phenon 4 (five strains of biovar 5; SUPP 1844, SUPP 20, MAFF 311043, MAFF 311151 and MAFF 302132). Group II (*D. dadantii*) corresponded to phenon I (five strains of biovar 3; MAFF 301767, SUPP 2200, SUPP 877, SUPP2162 and MAFF 106634). Group III (*D. dianthicola*) corresponded to phenon 5 [five strains of biovar 1 (MAFF 302984, SUPP 215, SUPP 2565, MAFF 311041, MAFF 311042), one strain of biovar 9 (MAFF 311149) and one strain of biovar 1* (SUPP 2525)]. Group IV (*D. zeae*) corresponded to phenon 1 (four strains of biovar 3 (MAFF 106502, SUPP 739, SUPP 410 and SUPP 1158) and two strains of biovar 8 (MAFF 311098 and SUPP 27)]. Group V (putative new species 1) corresponds to phenon 1* [14 strains of biovar 3 (SUPP 2451, SUPP 40, SUPP 1034, SUPP 2737, SUPP 2735, SUPP 2586, MAFF 311171, MAFF 311172, SUPP 420, SUPP 1152, SUPP 2738, SUPP 1164, SUPP 1352 and SUPP 2739)] and phenon 1 [three strains of biovar 3 (SUPP 2736, SUPP 1399 and SUPP 1539)]. Group VI (putative new species 2) also corresponded to phenon 1* [one strain of biovar 3 (MAFF 301677)].

Discussion

The present taxonomic investigation on 41 Japanese *Dickeya* strains isolated from 24 plant species included molecular techniques, namely DNA sequence analysis of *recA*, *dnaX*, *rpoD* and *gyrB* genes, and 16S rDNA; PCR genomic fingerprinting; and PCR–RFLP of *recA*, *rpoD* and *gyrB*. The strains were essentially divided into six groups (groups I–VI), four to existing *Dickeya* species [*D. chrysanthemi* (group I), *D. dadantii* (group II), *D. dianthicola* (group III) and *D. zeae* (group IV)], and two new genetic groups of *Dickeya* strains were found and assigned as group V and group VI. Group V comprises 17 strains, that were isolated from taro (strains SUPP 2586, MAFF 311171 and MAFF 311172), Welsh onion (SUPP 2451 and SUPP 40), *Phalaenopsis* (SUPP 1034, SUPP 2735, SUPP 2737 and SUPP 2736), *Cattleya* (SUPP 1399 and SUPP 2739), *Oncidium* (SUPP 1152 and SUPP 2738), *Dracaena* (SUPP 1352), *Vanda* (SUPP 1164), iris (SUPP 1539), and group VI consists of one strain, isolated from potato (MAFF 301677).

Sequence analysis of *rpoD* showed that these two new groups (group V and VI) differ from the existing *Dickeya* species (Supplementary Figure 2). Moreover, on the basis of the *dnaX* gene sequence analysis, which included the reference data for six other *Dickeya* strains, group V and group VI cannot be placed with any existing *Dickeya* species (Supplementary Figure 1). In the *recA* gene sequence analysis, again, these two groups could not be placed with the six known *Dickeya* species (Fig. 1).

On the basis of a *recA* sequence analysis, Parkinson et al. (2009) described three DUCs (DUC-1, DUC-2 and DUC-3). DUC-1 contains strains that were isolated from potato by Slawiak et al. (2009). DUC-2 consists of seven strains that were isolated from orchids (including *Phalaenopsis* and *Colocasia*), banana and yucca. Meanwhile, DUC-3 consists of only one strain, isolated from *Aglaonema*. None of the Japanese strains corresponded to DUC-1. In our study with *recA*, the strains within group V (putative new species 1), except SUPP 1034 (*Phalaenopsis*) and SUPP 2736 (*Phalaenopsis*), were placed in the same group as DUC-2. The two exceptions we found were placed in the same group with DUC-3. MAFF 301677 isolated from potato (group VI) cannot be placed within any of the DUC groups (Fig. 1). The type strain of *D. dadantii* analyzed in this study (NCPPB 898 acc. FJ216970) was placed independent of *D. dadantii*. We confirmed the Parkinson et al. (2009) suggestion that this *D. dadantii* type strain is atypical.

In the *gyrB* sequence analysis, the group VI strain (MAFF 301677) was placed with group III (*D. dianthicola*) (Supplementary Figure 3). The *gyrB* sequence was not useful for distinguishing group VI (MAFF 301677) from *D. dianthicola*.

In the 16S rDNA sequence analysis, the strain isolated from carnation (SUPP2525) and one from *Kalanchoe* (MAFF 311149) formed a clade independent of the other known *Dickeya* spp. groups (Supplementary Figure 4). However, in the *gyrB*, *rpoD* and *recA* sequence analyses, these two strains were grouped within group III, i.e., *D. dianthicola*. On the basis of biochemical tests, these strains were put in phenon 5, a phenon group of *D. dianthicola*. Thus, we concluded that these two strains are *D. dianthicola*. Hence, from the results of the 16S rDNA sequence analysis, we assigned these two strains as group III*.

The *dnaX* (IPO 2131 = CFBP 2052) and 16S rDNA (CFBP 2052) of the *D. zeae* type strain were grouped separately from the other *D. zeae* strains (Supplementary Figures 1 and 4). When the data for the other reference strains of *D. zeae* were included with the data for the 16S rDNA (BC 2877) and *dnaX* (IPO 649, IPO 650 and IPO 651) sequence analyses, the type strain was still placed in a different group from those additional reference strains of *D. zeae*. In contrast, the BC 2877, IPO 651, IPO 649 and IPO 650 strains were placed in the same group with Japanese *D. zeae*. When SR strains previously identified as *E. chrysanthemi* pv. *zeae* (SR 90, 120, 171 and 261) (Goto 1979) were added to the *dnaX* sequence analysis, again these strains were placed in the same group with Japanese *D. zeae* strains, suggesting that the sequence data of *D. zeae* type strain (CFBP 2052 = IPO 2131) might be inappropriate.

PCR–RFLP on specific loci has been used as a tool to differentiate various groups of bacteria (Olive and Bean 1999) as well as bacteria within the genus *Erwinia* including soft rot erwinias (*Pectobacterium* and *Dickeya*). Waleron et al. (2002) reported that diversity of bacteria within the former *Erwinia* genus including *Dickeya* spp. can be investigated using *recA* in the PCR–RFLP. It has also been reported that *E. amylovora* can be distinguished from other *Erwinia* strains using PCR–RFLP of *gyrA*, *rpoS* and *recA* (Toth et al. 2001), and Waleron et al. (2008) investigated the diversity of [illegible] soft rot erwinias using intergenic transcribed spacer (ITS) PCR–RFLP. The ITS PCR–RFLP also can be used to differentiate *P. carotovorum* (=*E. carotovora*) ssp. "*brasiliensis*" from another group of *Pectobacterium* species. (Duarte et al. 2004; van der Merwe et al. 2010).

Although the aforementioned PCR–RFLP analysis methods can be used to differentiate groups of bacteria, the results are difficult to analyze because so many band patterns are produced. In addition, restriction enzymes were randomly selected, and only one restriction enzyme was used for each gene was used in a single application, not in combination.

In this study, we developed a unique, locus-specific PCR–RFLP method. Using the results of a sequence

analysis of *gyrB*, *rpoD* and *recA*, we selected appropriate restriction enzymes for PCR–RFLP and used them in combination. Therefore, this PCR–RFLP method produced fewer band patterns; consequently, the results are much easier to analyze. The bands produced by each group of Japanese *Dickeya* spp. in this study can be used as a reference for using this PCR–RFLP to identify other *Dickeya* spp.

With the PCR–RFLP of *gyrB*, *rpoD* and *recA*, each group of the Japanese *Dickeya* strains can be clearly distinguished. In addition to the 41 *Dickeya* spp. strains used in this study, we can also identify 74 additional *Dickeya* (=*E. chrysanthemi*) strains of MAFF collection very rapidly (Supplementary Table 1). This method can thus be used as a rapid, easy technique to identify and distinguish *Dickeya* strains. It is also easier to do the technique and analyze the results compared with rep PCR techniques. However, to avoid misinterpretation, representative strains must be included. Furthermore, this method can only be used for the first step of identification. In this case, analyzing all three genes with PCR–RFLP and comparing the results will provide a more accurate identification. For more detailed information on the identity of strains, gene sequencing and phenotypic characterization should be also performed.

By means of *recA*, *rpoD* and *gyrB* PCR–RFLP, the SR strains that were isolated from corn (SR 90, 120, 171, 261), essentially gave the same RFLP pattern as the Japanese *D. zeae* strains, while SR 149, isolated from sugar cane, produced a different RFLP pattern from Japanese *D. zeae* or the other groups of the Japanese *Dickeya* strains (Supplementary Figures 5, 6, 7). In the *dnaX* sequence analysis, SR149 was placed in group independent of the six existing *Dickeya* species (Supplementary Figure 1). The *recA* sequence of SR 149 had 100 % identity with that of NCPPB 569 (FJ217027) (Fig. 1). NCPPB 569 was considered as a "new species level clade" by Parkinson et al. (2009). Thus, this SR 149 may also be included in the new species level clade of *Dickeya*. This new species level clade reported by Parkinson et al. (2009) is different not only from DUC-1, DUC-2, or DUC-3 but also from groups V and VI reported in this study. Both SR 149 and NCPPB 569 were isolated from sugar cane in Australia. More *Dickeya* isolates from sugarcane should be checked for their identity.

Based on their biochemical characteristics, *Dickeya* spp. (=*E. chrysanthemi*) has been grouped into six phena (phenotypic group) by Samson et al. (2005) and nine biovars by Ngwira and Samson (1990). Phenon 1 (all the members of biovar 3 and 8) corresponds to *D. zeae* and *D. dadantii*. Phenon 2 (all the members of biovar 6) and phenon 4 (all members of biovar 5) correspond to *D. chrysanthemi*. Phenon 5 (all members of biovar 1, 7 and 9) corresponds to *D. dianthicola*. Phenon 3 (all the members of biovar 2) corresponds to *D. dieffenbachiae*, and phenon 6 (all the members of biovar 4) corresponds to *D. paradisiaca*. In general, our results of biochemical tests correlated to the phenotypic characteristics described by Ngwira and Samson (1990) and Samson et al. (2005). However, on the basis of inulin utilization, five strains (MAFF 302984, SUPP 215, SUPP 2565, MAFF 311041 and MAFF 311042) from biovar 1, one strain (MAFF3 11149) from biovar 9, one strain (SUPP 2525) from biovar 1*, and one strain (SUPP 1844) from biovar 5 were negative for the utilization of inulin (Supplementary Table 2). Slawiak et al. (2009) also found that biovars 1 and 7 were negative for inulin utilization. Furthermore, Cother et al. (1992) also placed the strains that were negative for inulin into groups *D. dianthicola* or *D. chrysanthemi*. Therefore, as stated by Slawiak et al. (2009), we concluded that the inulin utilization test cannot be used to differentiate *D. dianthicola* from other *Dickeya* species.

All the phenon and biovar characteristics reported by Samson et al. (2005) and Ngwira and Samson (1990) correspond to the species of *Dickeya* with one exception. Phenon 1 (all the members of biovars 3 and 8), in particular biovar 3, was previously reported to correspond to either *D. zeae* or *D. dadantii*. Recently, putative new species of *Dickeya* that belong to biovar 3 have also been found (groups V and VI in this study; Parkinson et al. 2009; Slawiak et al. 2009). Thus, phenon 1 and biovar 3 do not always correspond to *D. zeae* and *D. dadantii*.

Brady et al. (2012) proposed *D. dieffenbachiae* as a subspecies of *D. dadantii* and named it *D. dadantii* ssp. *dieffenbachiae*. In this study, we could not find any *D. dieffenbachiae* strains among Japanese *Dickeya* strains. Here, we continue using the species name *D. dieffenbachiae* as a species separate from *D. dadantii*. In our opinion, we need more convincing proof for grouping *D. dieffenbachiae* in the group of *D. dadantii*.

This study showed that some of the Japanese *Dickeya* strains can be distinguished by sequencing analysis but cannot always be distinguished by phenotypic tests. In contrast, one carnation strain (SUPP 2525) can be distinguished by phenotypic tests from members of group III (biovars 1 and 9; *D. dianthicola*) but cannot be distinguished by sequencing analysis.

Each method used in the present study [PCR genomic fingerprinting, multi locus sequence analysis (MLSA) and RFLP of *recA*, *rpoD* and *gyrB*] has advantages and disadvantages. PCR genomic fingerprinting seems to yield results easier and faster than MLSA can—within 7–8 h. However, because so many bands are produced, the results of PCR genomic fingerprinting are more difficult to analyze and thus can be misinterpreted. The results of MLSA may be easier to analyze and will give more precise results compared with rep PCR. Unfortunately, MLSA is expensive and takes longer.

PCR–RFLP of *gyrB*, *rpoD* and *recA* is more rapid and efficient than PCR genomic fingerprinting and MLSA. It is easy to perform, easy to analyze, cheaper and quicker (only 5 h from start to finish). Because fewer bands are produced, it is much easier to analyze, and misinterpretations are less likely. Furthermore, the results of this method correspond well with the results of PCR genomic fingerprinting and MLSA (Table 3).

As for host plants, each species of the Japanese *Dickeya* strains has a diverse host range. *Dickeya chrysanthemi* (group I) found on chicory, chrysanthemum and eggplant. *D. dadantii* (group II) has been found on sweet potato, carrot, eggplant, peach and strawberry. *Dickeya dianthicola* (group III) has been found on yacon, carnation, *Kalanchoe*, potato and chrysanthemum. *D. zeae* (group IV) has been found on corn, rice, *Calanthe* and setaria. *Dickeya* putative new species 1 (group V) has been found on Welsh onion, taro, *Phalaenopsis*, *Cattleya*, bush lily, *Dracaena*, *Iris*, *Oncidium* and *Vanda*. Meanwhile, *Dickeya* putative new species 2 (group VI) has only been found on potato. In addition, a single host plant can be infected by one or more *Dickeya* species, which means that the identity of a *Dickeya* species does not always correspond to a particular host species. Thus, we cannot predict the identity of the *Dickeya* species using host plants; for accurate identification, we need to determine the genetic characteristics of the isolates.

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