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Application of RFLP analysis of *recA*, *gyrA* and *rpoS* gene fragments for rapid differentiation of *Erwinia amylovora* from *Erwinia* strains isolated in Korea and Japan

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Abstract Restriction fragment length polymorphism (RFLP) analysis of the PCR amplified fragments of *recA*, *gyrA* and *rpoS* genes was applied for the characterization of *Erwinia amylovora* and *Erwinia* strains, which cause fire blight and Asian pear blight in orchards. Primers, constructed on the basis of the published *recA, gyrA* and *rpoS* gene sequences of *Erwinia carotovora*, allowed us to amplify DNA fragments for RFLP differentiation of *E. amylovora* and *E. pyrifoliae* and finally to distinguish strains within these species and relate them to pear pathogens from Japan. Three to seven restriction endonucleases were applied for RFLP analysis of each gene fragment. The electrophoretic patterns generated after PCR–RFLP for each of the tested genes, were characteristic and specific for each species and allowed their differentiation. The data show that PCR–RFLP analysis of the *recA, gyrA* and *rpoS* gene fragments may be considered as a useful tool for the identification and differentiation of *E. amylovora* and *E. pyrifoliae*. Almost identical restriction patterns of the analyzed gene fragments indicated a high relationship of *E. pyrifoliae* strains from Korea and pear pathogens from Japan and a divergence to *E. amylovora*. For quick and effective differentiation of *E. amylovora* strains from *Erwinia* strains from Asia without nucleotide sequencing we recommend the amplification of *recA* and *rpoS* gene fragments and digestion of each of them with restriction endonuclease *Hin*6I.

Keywords PCR fingerprinting · Plant pathogenic bacteria · Housekeeping genes

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Introduction

Fire blight is a destructive bacterial disease, caused by *Erwinia amylovora* and affects many species of rosaceous plants of economic importance, including fruit trees and ornamentals (Vanneste 2000). It is responsible for large losses in the production of pome fruits in North America, Europe, New Zealand and other countries of the world. With regard to many criteria, *E. amylovora* is a homogeneous species. There are no major differences in biochemical characters, carbohydrate utilization (Dye 1968), or protein electrophoretic characteristics (Vantomme et al. 1982) among *Erwinia amylovora* strains despite

their different geographical and host origins. This homogeneity has also been confirmed with serological (Vantomme et al. 1982), host range and genetic studies (Vanneste 1995). *Erwinia amylovora* strains were distinguished by analysis of short sequence DNA repeats (SSR) (Kim and Geider 1999; Jock et al. 2003; Ruppitsch et al. 2004), and by random DNA amplification techniques such as RAPD (Brennan et al. 2002). Highly related fruit tree strains were distinguished from *Rubus* strains by RFLP analysis of the *hrp* genes (Laby and Beer 1992; Jock and Geider 2004), but also by PCR ribotyping (Beer et al. 1996; Maes et al. 1996; Momol et al. 1997). In addition, Pulsed Field Gel Electrophoresis (PFGE) has been found to be useful to classify strains of different geographical origins (Zhang and Geider 1997; Jock et al. 2002; Jock and Geider 2004).

Erwinia amylovora is the causative agent of fire blight in North America, Europe, New Zealand, a disease not described in Central and Eastern Asia, South America and South Africa. In Korea, the causative agent of bacterial shoot blight of pears is *E. pyrifoliae* (Kim et al. 1999; Rhim et al. 1999). In Japan a similar pathogen causing bacterial shoot blight on pears was tentatively designated as *E. amylovora* pv. *pyri* (Goto 1992). Microbiological tests, BIOLOG assays and PCR analyses suggested a close resemblance with *E. amylovora* (Kim et al. 1996), also suggested by DNA/DNA hybridization analysis (Mizuno et al. 2000). However, the comparison of DNA sequences from genes coding EPS or harpin synthesis and analysis of the 16S rRNA intergenic transcribed spacer (ITS) showed that this pathogen is more related to *E. pyrifoliae*, than to *E. amylovora* (Kim et al. 2001; Jock and Geider 2004). This notion was supported by AFLP fingerprinting analysis of *groEL* genes and plasmid profiling (Maxson-Stein et al. 2003).

A new approach, multi-locus sequence analysis (MLSA), based on the application of several molecular markers, provides more information about genome specificity and can therefore help to identify bacteria and to study their genetic diversity and phylogeny (Maiden et al. 1998). Apart from 16S and 23S rDNA, there are several other molecular markers which can serve for the identification and differentiation of bacterial pathogens, such as: *groEL*, *hsp*60, *recA, gyrA* and *rpoS* (Ludwig and Schleifer 1999). Our earlier results indicate the usefulness of *recA* and *rpo*S PCR-RFLP analysis for genotyping of species from the former *Erwinia* genus (Waleron et al. 2002a, b).

The goal was to determine whether molecular markers based on PCR–RFLP analysis of *rec*A, *gyr*A and *rpo*S genes can be effectively used for the differentiation of *E. amylovora* from other *Erwinia* species causing fruit tree diseases. In addition it is a primary step to check if the polymorphism of housekeeping genes (*recA*, *gyrA*, *rpoS*) is sufficient to use them for MLSA of *Erwinia* species in the future.

Materials and methods

Bacterial strains

Nineteen strains of *E. amylovora*, six strains of *E. pyrifoliae* from Korea and six *Erwinia* spp. strains from Japan were used in this study (Table 1). In addition, strains from seven other species of *Erwinia* (*E. billingiae*, *E. mallotivora, E. persicina, E. psidii, E. rhapontici, E. tasmaniensis* and *E. tracheiphila*) were used for comparative purposes. For DNA preparation, bacterial strains were grown over night at 28°C in Luria Broth medium. Cells were harvested by centrifugation and resuspended in TE buffer (50 mM Tris–HCl, 40 mM EDTA, pH 8.0). The total genomic DNA was extracted using the SDS/proteinase K based method (Ausubel 1992).

Primer design and PCR amplifications

Oligonucleotide primers were designed on the basis of the sequence of *Pectobacterium carotovorum gyrA, recA*, and *rpoS* genes available in GenBank. The sequences of primers shown in Table 2 were checked for homology to other sequences, which may also be amplified, in GenBank and EMBL databases using the BLAST-n programme.

DNA amplification was performed in 50 µl reaction volumes containing 5 µl of 10× reaction buffer (Fermentas), 2.0 mM $MgCl_2$, 250 mM each of dATP, dCTP, dGTP and dTTP, 20 pmol of each primer, 0.1% (*v*/*v*) Tween 20, 50–100 ng of DNA and 1 U of recombinant Taq DNA polymerase (Fermentas). Amplification was performed using a UNOII Biometra thermocycler, with initial denaturation (95°C, 3 min), followed by 32 cycles of denaturation (94°C, 1 min), annealing (47°C, 1 min for *rec*A, 56°C, 1 min

Table 1 Origin and recA, gyrA, and rpoS PCR–RFLP groups of the bacterial strains used in this study

Bacterial strains	Host	Geographic origin	RFLP groups based on		
			recA[a]	*gyrA*	*rpoS*
Erwinia amylovora					
ATCC 15580[Ti]	Pear	UK	32	1	1
Ea394[b]	Pear	Poland	32	1	1
Ea661[b]	Rowan Poland	32	1	1	
Ea684[b]	*Crataegus* sp.	Poland	32	1	1
Ea691[b]	Apple	Poland	32	1	1
Ea840[b]	Pear	Poland	32	1	1
Ea1056[c]	Quince	Greece	32	1	1
Ea1628[c]	Quince	Bulgaria	32	1	1
Ea7001M[d]	Mountain ash	Canada	32	1	1
EaA8[f]	Unknown	unknown	32	1	1
EaG-5[d]	Pear or Apple	Canada	32	1	1
EaG-7[d]	Pear or Apple	Canada	32	1	1
EaIL6[e]	Raspberry	Illinois	32	1	1
EaKa6[e]	Apple	Canada	32	1	1
EaKp1/00[e]	Pear	Canada	32A	1	1
EaP-1[d]	Pear or Apple	Canada	32A	1	1
EaRUB7[e]	Raspberry	Canada	32A	1	1
CFBP1232[Te]	Pear	England	32	1	1
LMG2024		32	1	1	
Erwinia pyrifoliae					
Ep1/96[e]	Asian pear	South Korea	58	2	3
Ep4/97[e]	Asian pear	South Korea	58	2	3
Ep8/95[e]	Asian pear	South Korea	58	2	3
Ep16/96[Te]	Asian pear	South Korea	58	2	2
Ep28/96[e]	Asian pear	South Korea	58	2	3
Ep31b/96[e]	Asian pear	South Korea	58	2	3
Erwinia spp.					
Ejp546[e]	Asian pear	Japan	59	no	3
Ejp547[e]	Asian pear	Japan	59	no	3
Ejp556[e]	Asian pear	Japan	58	3	4
Ejp557[eT]	Asian pear	Japan	59	no	3
Ejp562[e]	Asian pear	Japan	59	no	3
Ejp617[e]	Asian pear	Japan	60	3	5
Erwinia billingiae					
Eb661[e] (NCPPB 661)	Pear	England	61	no	6
Erwinia mallotivora					
CFBP 2503[Th]	*Mallotus japonicus*	Japan	55	no	7
Erwinia persicina					
ATCC 35998[Ti]	Tomato	Japan	49	no	8
ATCC 10756[g]	Human	Texas, USA	63	no	9
Erwinia psidii					
CFBP 3627[h]	Common guava	Brazil	54	4	10

Table 1 (continued)

Bacterial strains	Host	Geographic origin	RFLP groups based on		
			recA[a]	*gyrA*	*rpoS*
Erwinia rhapontici					
CFBP 3618[e]	Rhubarb	UK	53	5	11
Erwinia tasmaniensis					
Et1/99[Te]	Apple	Tasmania, Australia	62	6	12
Et2/99[e]	Pear	Victoria, Australia	62	6	13
Erwinia tracheiphila					
ATCC 119321[Te]	Wheat	USA	57	no	14

The superscripted T represents the type strain; ATCC 15580 and CFBP1232 are the *E. amylovora* type strain from different collections.

no A PCR product was not obtained.

[a] RFLP groups according to classification in Waleron et al. 2002a

[b] P. Sobiczewski, Institute of Pomology, Skierniewice, Poland

[c] P.G. Psallidas, Benaki Phytopathological Institute, Kifissia, Athens, Greece

[d] D. M. Hunter, Tree Fruits Agriculture and Agri-Food Canada Southern Crop Protection & Food Research Centre, Canada

[e] Geider et al. 2006, Jock and Geider 2004, and Kim et al. 2001

[f] D. Coplin, Ohio State University Columbus, Ohio, USA

[g] C. M. O'Hara, Centre for Disease Control and Prevention, Atlanta, Georgia, USA

[h] L. Gardan, Institute de la Recherche Agronomique Centre de Recherches Agronomique d'Angers, Angers, France

[i] S.-W. Kwon, National Institute of Agricultural Science & Technology, RDA, Suwon, Korea

for *gyr*A and 55°C, 1 min for *rpo*S), and extension (72°C, 2 min), with a final extension (72°C, 5 min). The amplified products were electrophoretically separated in a 6% (*w*/*v*) polyacrylamide gel at 120 V for 10 h or in 1. 5% (*w*/*v*) agarose gel at 75 V for 2 h in 1× Tris-borate EDTA (TBE) buffer, pH 8.3 and visualized with UV light after staining in ethidium bromide (0.5 μg ml^{-1}). The amplification of all three genes was possible directly from cells added to the PCR reaction instead of isolated DNA. Lysis in 0.1% Tween for 15 min at 65°C increased sensitivity. This modified PCR assay significantly reduced time and costs of the identification procedure for bacteria.

Restriction fragment length analysis

The amplified DNA fragments of *gyrA, recA* and *rpoS* genes were digested with restriction endonucleases selected on the basis of the nucleotide sequence of the studied genes using Vector NTI software. The amplified fragments of the *rec*A gene were digested with *Alu*I, *Bsu*RI, *Fnu*DII, *Hin*6I, *Hin*fI, *Tas*I, and *Tru*1I restriction endonucleases, *gyr*A with *Csp*6I, *Msp*I and *Sau*3A and the amplified fragments of *rpo*S with *Alu*I, *Hin*6I, *Hin*fI and *Tru*1I. All applied restriction endonucleases were purchased from Fermentas. Restriction analysis was performed overnight with 2.5 U of each endonuclease

Table 2 Sequences of PCR primers used in this study

Primer name	Target gene	Sequences (5′-3′)	Length (bases)	Reference
recA1	*recA*	GGTAAAGGGTCTATCATGCG	20-mer	Waleron et al. 2002a
recA2		CCTTCACCATACATAATTTGGA	22-mer	Waleron et al. 2002a
gyrA1	*gyrA*	TGGTGACGCGTCGTACCATT	20-mer	Waleron 2002c
gyrA4		GCAGAGAACAGCATCGCTTC	20-mer	Waleron 2002c
rpoS1	*rpoS*	ATGAGCCAAAGTACGCTGAA	20-mer	Waleron et al. 2002b
rpoS2		ACCTGAATCTGACGAACACG	20-mer	Waleron et al. 2002b

using the buffer and temperature recommended by the manufacturer (Fermentas). Restriction fragments were separated in a 12% (*w*/*v*) polyacrylamide gel at 120 V for 10 h in 1× TBE buffer and visualized with UV light after staining in ethidium bromide (0.5 μg ml^{-1}).

Data analysis

Following electrophoresis of polyacrylamide gels, images were digitized and band profiles were analyzed using software: Bionumerix v 4.05 (Applied Maths, Kortrijk, Belgium). The Pearson product–moment correlation coefficient was used to estimate levels of similarity between profiles for each strain. Unweight pair-group method of averages (UPGMA) algorithm was used to construct dendrograms from similarity matrices.

Results

Comparison of *recA* gene fragments amplified from the genome of *E. amylovora* and other *Erwinia* strains

DNA isolated from the cells of pre-screened *Erwinia* strains was used as a target in the PCR reactions. The primers used for the amplification of the *recA* gene fragment (Waleron et al. 2002a, c) generated amplification products estimated at 870 bp for all of the *E. amylovora* strains and 800 bp products for *E. pyrifoliae* strains, as determined on the basis of non-denatured polyacrylamide electrophoresis (data not shown). This peculiar migration of amplified *recA* gene fragments of *E. amylovora* and of *E. pyrifoliae* could even be used to distinguish both species.

PCR products were digested by seven restriction endonucleases: *Alu*I, *Bsu*RI, *Fnu*DII, *Hin*6I *Hin*fI, *Tas*I, and *Tru*1I in separate reactions. The application of three endonucleases: *Fnu*DII, *Hin*6I and *Tru*1I enabled us to distinguish the examined species (Fig. 1a–c and Table 3). The other four endonucleases: *Alu*I, *Bsu*RI, *Hinf*I and *Tas*I gave similar electrophoretic patterns for DNA fragments amplified from the genome of both species. Restriction analysis of the *rec*A gene allowed the description of five RFLP groups for the *E. amylovora, E. pyrifoliae* and the *Erwina* spp. from Japan (Table 1 and Fig. 1). The numbers of RFLP groups corresponded with those described earlier (Waleron et al. 2002a). Two groups (32 and 32A) were characteristic for *E. amylovora* strains. Group 58 was unique for six *E. pyrifoliae* strains from Korea and one *Erwinia spp.* strain from Japan (Ejp556), while groups 59 and 60 were typical only for *Erwinia* spp. from Japan (Table 1 and Fig. 1).

The obtained pattern indicated high homogeneity within *E. amylovora*. Only the application of a restriction endonuclease *Fnu*DII allowed us to differentiate the *E. amylovora* strains into two RFLP groups (Fig. 1c). The first profile (32) was characteristic for 16 out of the 19 *E. amylovora* strains examined. In the case of *E. pyrifoliae*, all isolates from Korea were identical according to restriction analysis of the *rec*A gene fragment. Japanese *Erwinia* spp. strains were more heterogeneous. Endonuclease *Tru*1I allowed the description of three RFLP patterns (Fig. 1 and Table 3). Strain Ejp556 gave patterns characteristic for *E. pyrifoliae*. Four strains (Ejp546, Ejp547, Ejp557 and Ejp556) had identical patterns, different from strain Ejp617 (Fig. 1 and Table 3).

Comparison of *gyrA* gene fragments amplified from *E. amylovora* and other *Erwinia* strains

The primers for the *gyr*A gene were designed on the basis of the *Erwinia carotovora gyr*A gene cloned by (Rosanas et al. 1995). The amplification product of about 970 bp was obtained for all tested *E. amylovora* and *E. pyrifoliae* strains and for two *Erwinia* spp. strains from Japan (Ejp556 and Ejp617; Table 1). PCR products were digested by three restriction endonucleases: *Csp*6I, *Msp*I and *Sau*3A in separate reactions. All restriction endonucleases gave single, characteristic RFLP patterns for each group: *E. amylovora, E. pyrifoliae* and *Erwinia* strains from Japan (Fig. 2a–c and Table 3).

Comparison of *rpoS* gene fragments amplified from *E. amylovora* and other *Erwinia* strains

The primers designed for the *rpoS* gene of *E. amylovora* OT1 available in GenBank generated an amplification product of about 880 bp either for *E. amylovora, E. pyrifoliae* or for *Erwinia* spp. strains from Japan. Only in the case of *E. pyrifoliae* Ep 16/96 was the obtained product smaller, and had a size of about 780 bp indicating a deletion in the *rpoS* gene.

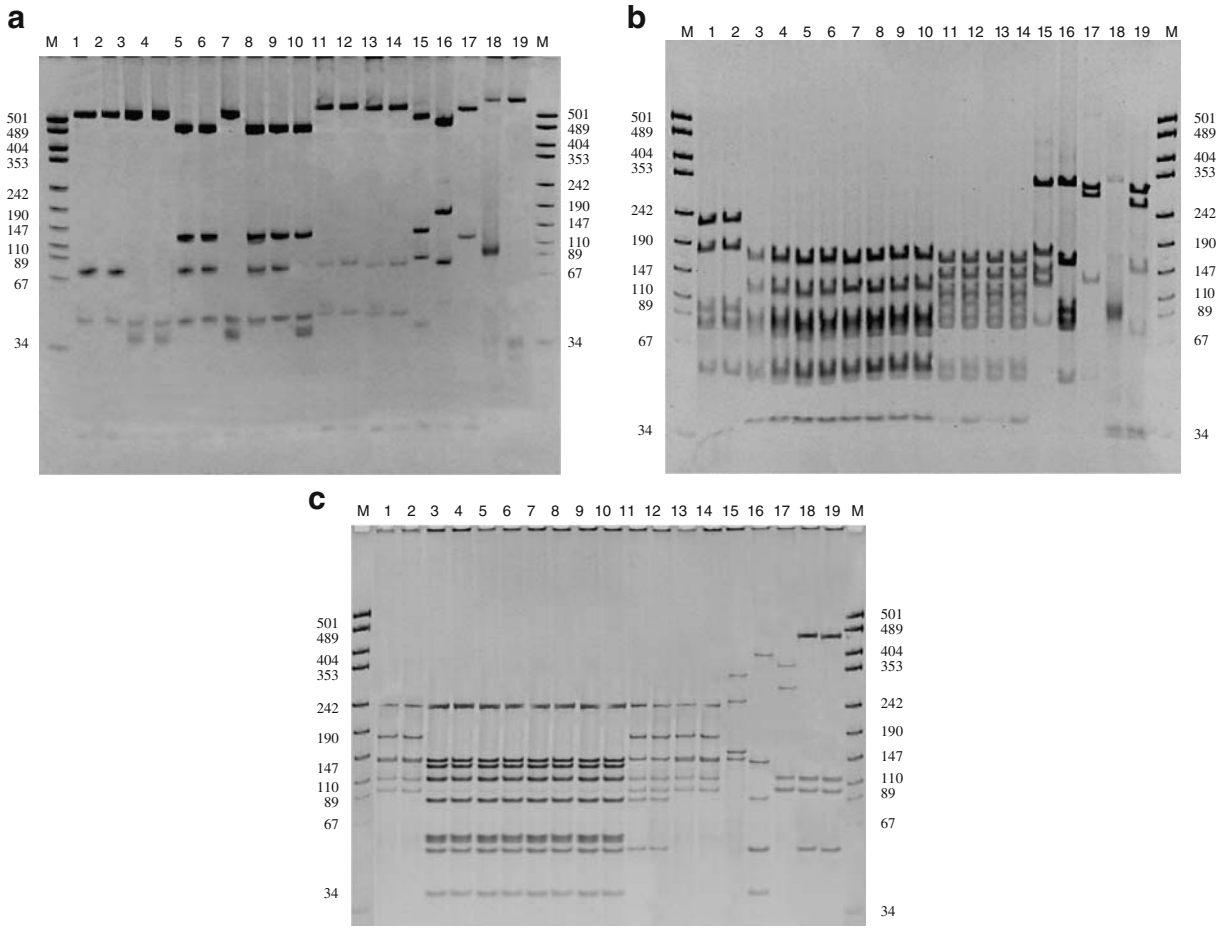

Fig. 1 Restriction analysis of *recA* gene fragments amplified by PCR. RFLP patterns obtained after digestion of PCR products with the following endonucleases: **a** *Tru*1I, **b** *Hin*6I, **c** *Fnu*DII. Lanes: M, molecular size markers (pUC19/*Msp*I, Fermentas); 1, *E. tasmaniensis* Et1/99; 2, *E. tasmaniensis* Et2/99; 3, *E. pyrifoliae* Ep16/96; 4, *E. pyrifoliae* Ep1/96; 5, *Erwinia* sp. Ejp546; 6, *Erwinia* sp. Ejp547; 7, *Erwinia* sp. Ejp556; 8, *Erwinia* sp. Ejp557; 9, *Erwinia* sp. Ejp562; 10, *Erwinia* sp. Ejp617; 11, *E. amylovora* Ea394; 12, *E. amylovora* CFBP1232; 13, *E. amylovora* EaP-1; 14, *E. amylovora* Ea Kp1/00; 15, *E. psidii* CFBP 3627; 16, *E. mallotivora* CFBP 2503; 17, *E. billingiae* Eb 661; 18, *E. rhapontici* CFBP3618; 19, *E. persicina* ATCC 35998

The PCR products were digested with five restriction endonucleases: *Alu*I, *Fnu*DII, *Hin*6I, *Hin*fI and *Tru*1I in separate reactions. RFLP analysis allowed us to describe five restriction groups (Tables 1 and 3 and Fig. 3). RFLP group 1 was unique for *E. amylovora* strains. The second RFLP group was characteristic only for one *E. pyrifoliae* strain Ep16/96. The restriction pattern of the *rpo*S gene fragment of *E. pyrifoliae* Ep16/96 showed a deletion of a fragment approximately 140 bp (Fig. 3a–d and Table 3). Other strains of *E. pyrifoliae* and the four Japanese *Erwinia* spp. strains belonged to the third RFLP group. RFLP groups 4 and 5 were described only for a single *Erwinia* spp. strain from Japan (Tables 1 and 3; Fig 3).

Compilation of RFLP analysis of *recA* and *rpoS* genes

A consensus dendrogram was constructed on the basis of RFLP patterns of *recA* and *rpo*S genes from four *E. amylovora* strains, six *E. pyrifoliae*, four *Erwinia* strains from Japan, and type strains from other *Erwinia* species: *E. billingiae*, *E. mallotivora*, *E. psidii*, *E. persicina*, *E. rhapontici* and *E. tasmaniensis*. A consensus dendrogram revealed the presence of at least three separated clusters (Fig. 4). One consisted

Table 3 RFLP groups obtained on the basis of restriction analysis of *recA, gyrA* and *rpoS* gene fragments with seven restriction endonucleases

RFLP[a] groups	PCR–RFLP patterns for specific restriction endonucleases							*Erwinia* species assigned	No. of strains
	*Tas*I	*Tru*1I	*Alu*I	*Hinf*I	*Fnu*DII	*Bsu*RI	*Hin*6I		
recA–PCR–RFLP									
32	*12*	*1*	*2*	*3*	*1*	*1*	*1*	*E. amylovora*	14
32A	*12*	*1*	*2*	*3*	*2*	*1*	*1*	*E. amylovora* KP1, P-1, RUB7	3
49	*26*	*14*	*2*	*1*	*10*		*7*	*E. persicina*	2
53	*30*	*14*	*2*	*3*	*8*		*6*	*E. rhapontici*	1
54	*31*	*13*	*9*	*3*	*6*		*5*	*E. psidii*	1
55	*0*	*6*	*9*	*3*	*5*		*4*	*E. mallotivora*	1
57	*23*	*9*	*2*	*1*	*9*		*9*	*E. tracheiphila*	1
58	*12*	*14*	*2*	*3*	*3*	*2*	*2*	*E. pyrifoliae* + Ejp 556	6 + 1
59	*12*	*1*	*2*	*3*	*3*	*2*	*2*	Ejp 546, 547, 557, 562	4
60	*12*	*15*	*2*	*3*	*3*	*2*	*2*	Ejp 617	1
61	*32*	*16*			*7*		*8*	*E. billingiae*	1
62	*33*	*1*			*4*		*3*	*E. tasmaniensis*	2
gyrA-PCR-RFLP									
	*Csp*6I			*Msp*I		*Sau*3AI			
1	*2*			*2*		*2*		*E. amylovora*	17
2	*13*			*1*		*1*		*E. pyrifoliae*	6
3	*3*			*1*		*1*		Ejp 556, Ejp 617	2
4	*6*			*6*		*6*		*E. psidii*	1
5	*7*			*7*		*7*		*E. rhapontici*	1
6	*8*			*8*		*8*		*E. tasmaniensis*	2
rpoS PCR-RFLP									
	*Hin*6I	*Hin*fI	*Alu*I	*Tru*1I	*Fnu*DII				
1	*1*	*1*	*1*	*1*	*1*			*E. amylovora*	17
2	*2*	*2*	*2*	*2*	*2*			*E. pyrifoliae* 16/96	1
3	*3*	*1*	*3*	*1*	*3*			*E. pyrifoliae* (5 of 6) + Ejp 556	6
4	*4*	*1*	*3*	*1*	*3*			Ejp546, 547, 557, 562	4
5	*3*	*1*	*3*	*3*	*3*			Ejp 617	1
6	*5*	*3*	*4*	*4*	*4*			*E. billingiae*	1
7	*6*	*5*	*5*	*5*	*5*			*E. mallotivora*	1
8	*12*	*10*	*10*	*10*	*11*			*E. persicina*	1
9	*13*	*11*	*11*	*11*	*12*			*E. persicina*	1
10	*7*	*6*	*6*	*6*	*6*			*E. psidii*	1
11	*11*	*9*	*9*	*9*	*10*			*E. rhapontici*	1
12	*8*	*7*	*7*	*7*	*7*			*E. tasmaniensis*	1
13	*9*	*7*	*7*	*7*	*8*			*E. tasmaniensis*	1
14	*10*	*8*	*8*	*8*	*9*			*E. tracheiphila*	1

Italic numbers correspond with RFLP patterns shown in Figs. 1, 2, and 3.

[a] Numbers of RFLP groups based on the combined PCR–RFLP patterns.

of *E. pyrifoliae* strains from Korea and *Erwinia* spp. strains from Japan; the second cluster grouped *E. amylovora* strains and the third cluster consisted of *E. tasmaniensis* strains. Other strains of *Erwinia* were grouped separately.

All *Erwinia* spp. strains from Japan were grouped in cluster 1, together with *E. pyrifoliae* strains (Fig. 4). Almost all *Erwinia* spp. strains from Japan were grouped together in sub-cluster 1a. Only one strain Ejp556 was grouped with *E. pyrifoliae* strains from

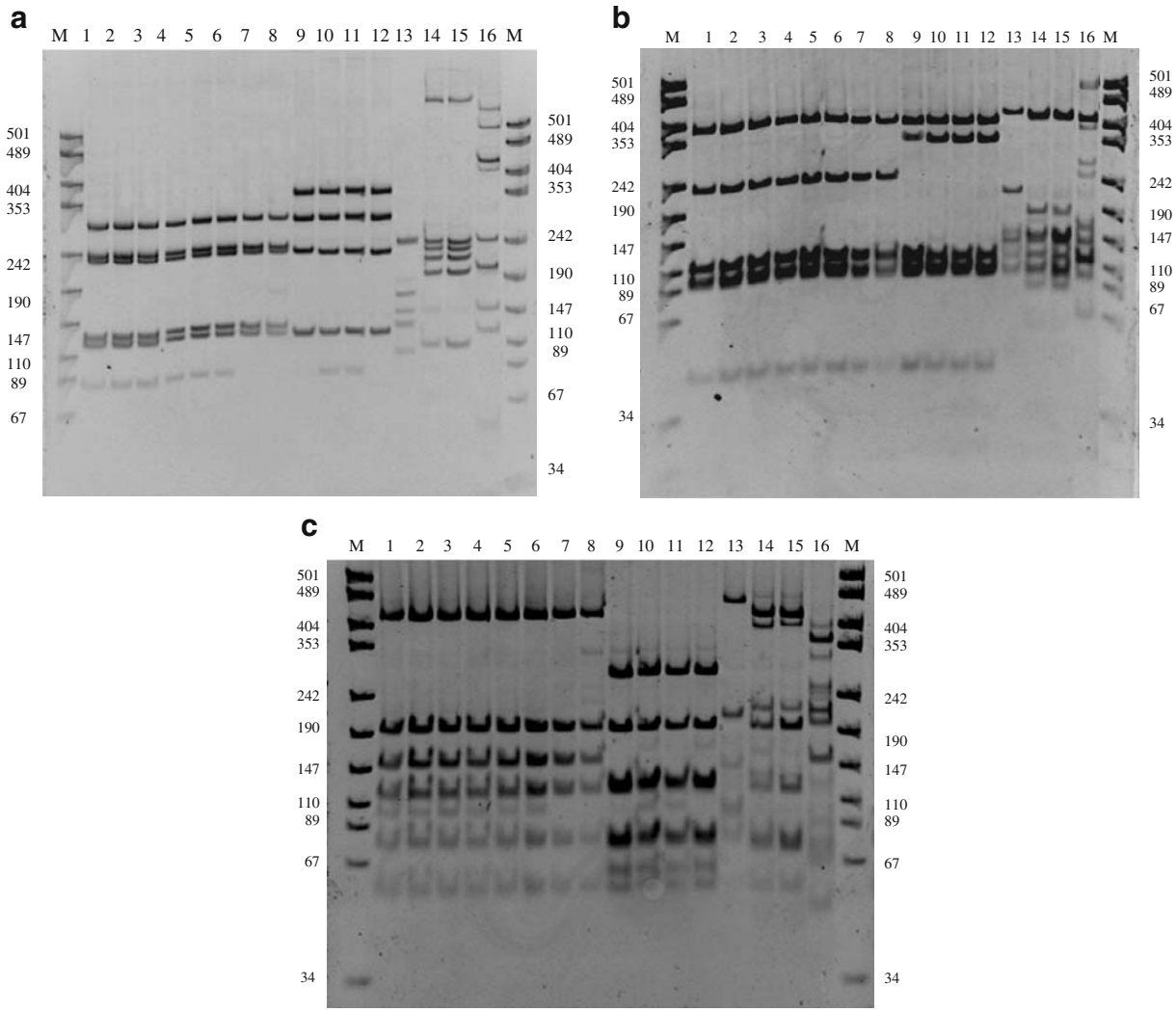

Fig. 2 Restriction analysis of the *gyrA* gene fragment amplified by PCR. RFLP patterns obtained after digestion of PCR products with the following endonucleases: **a** *Csp*6I, **b** *Msp*I, **c** *Sau*3*A*I. Lanes: M, molecular size markers (pUC19/*Msp*I, Fermentas); 1, *E. pyrifoliae E. pyrifoliae* Ep16/96; 2, *E. pyrifoliae* Ep4/97; 3, *E. pyrifoliae* Ep28/96; 4, *E. pyrifoliae* Ep316/96; 5, *E. pyrifoliae* Ep8/95; 6, *E. pyrifoliae* Ep4/97; 7, Ejp556; 8, Ejp617; 9, *E. amylovora* EaKp1/00; 10, *E. amylovora* CFBP 1232; 11 - *E. amylovora* Ea394; 12, *E. amylovora* EaP-1; 13, *E. rhapontici* CFBP 3618; 14, *E. tasmaniensis* Et1/99; 15, *E. tasmaniensis* Et2/99; 16, *E. persicina* ATCC 35998

Korea which were identical in RFLP analysis of all three tested genes and formed sub-cluster 1b. The third sub-cluster 1c consisted of only one *E. pyrifoliae* strain Ep 16/96 due to the deletion in the *rpoS* gene (Fig. 4).

Discussion

The amplified fragments of the three housekeeping genes *recA*, *gyrA* and *rpoS* from *E. amylovora*, *E. pyrifoliae* and *Erwinia* spp. strains from Japan were compared using PCR–RFLP. Our earlier work indicated the usefulness of *recA* and *rpoS* PCR–RFLP for identification of different species from the former genus *Erwinia* (Waleron et al. 2002a, b).

Amplification of the above gene fragments and later restriction analysis assigned characteristic RFLP patterns for the gene fragment and the species (Tables 1 and 3). In the case of *E. amylovora* strains, two different RFLP profiles for the *rec*A gene fragment were described and one of them was dominant. Isolates of *E. pyrifoliae* generated one RFLP profile for

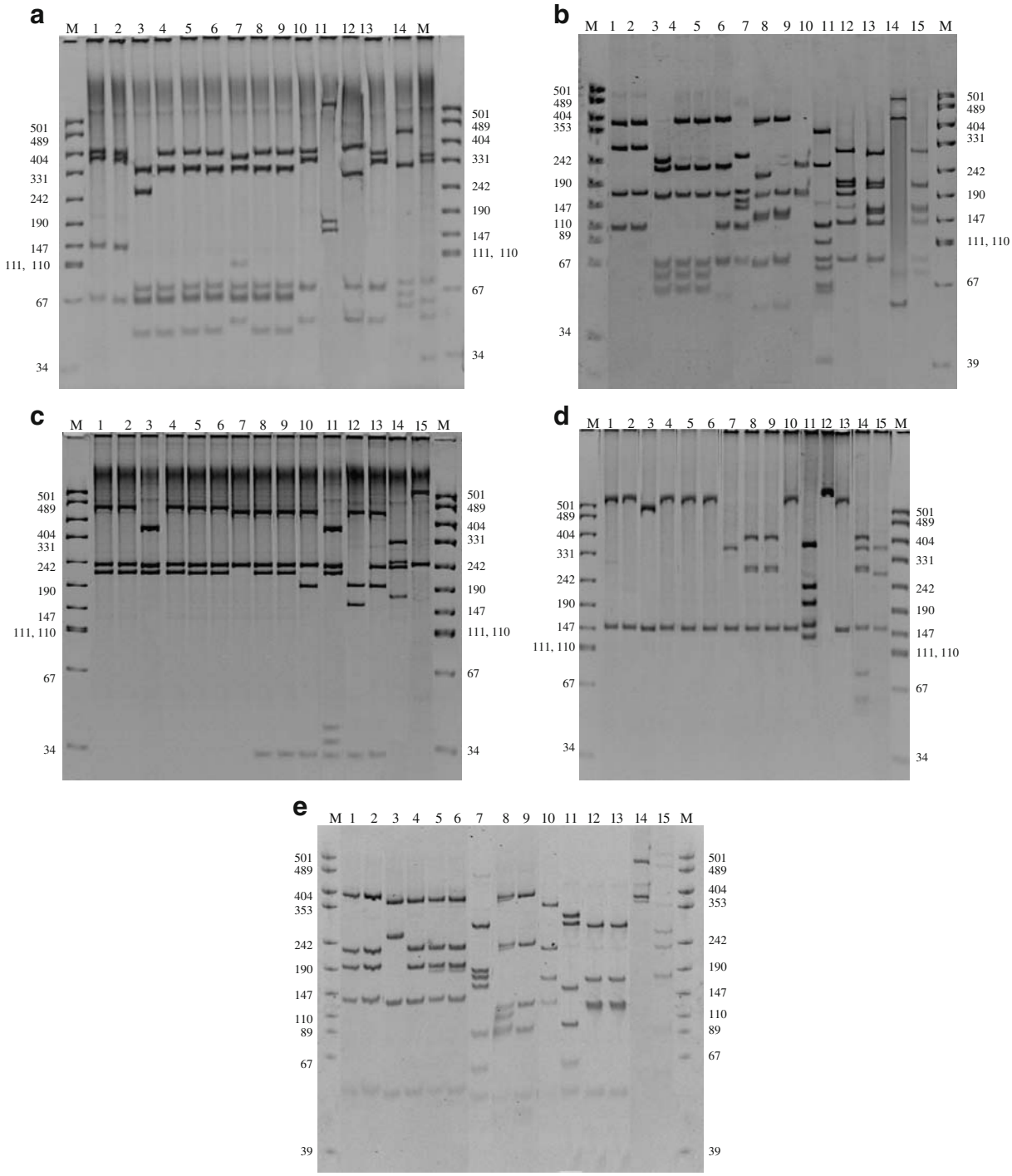

Fig. 3 Restriction analysis of the *rpoS* gene fragment amplified by PCR reaction. RFLP patterns obtained after digestion of PCR products with the following endonucleases: **a** *Alu*I, **b** *Hin*6I, **c** *Hinf*I, **d** *Tru*1I, **e** *Fnu*DII. Lanes: M, molecular size markers (pUC18/*Msp*I); 1, *E. amylovora* Ea394; 2, *E. amylovora* EaKp1/00; 3, *E. pyrifoliae* Ep16/96; 4, *E. pyrifoliae* Ep1/96; 5, Ejp556; 6, Ejp557; 7, *E. billingiae* Eb661; 8, *E. tasmaniensis* Et1/99; 9, *E. rhapontici* CFBP3618; 10, *E. tracheiphila* ATCC 119321; 11, *E. persicina* ATCC 35998; 12, *E. persicina* TCC 756; 13, *E. mallotivora* CFBP 2503;14, *E. psidii* CFBP 3627

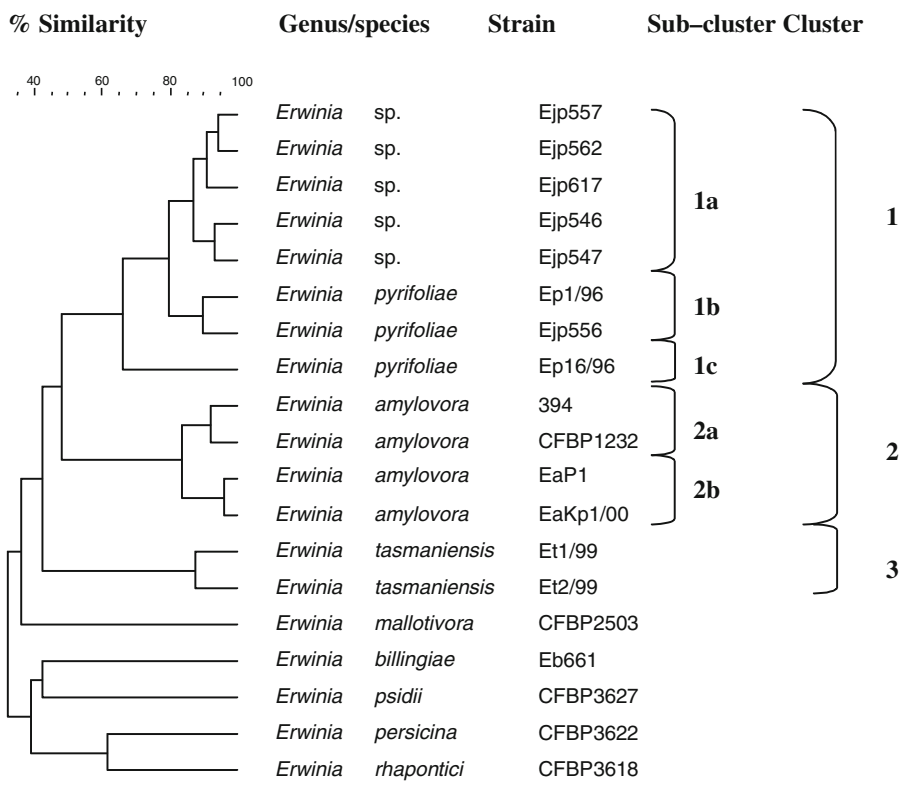

Fig. 4 A consensus dendrogram derived from the UPGMA linkage of correlation coefficients for *recA* and *rpoS* RFLP profiles from species in the genus *Erwinia*. The level of linkage is expressed in percentage. Other *E. amylovora* strains except Ea RUB7 have the same pattern as *E. amylovora* Ea394; Ea RUB7 has an identical pattern to EaKp1/00, other *E. pyrifoliae* strains have the same pattern to *E. pyrifoliae* Ep1/96

recA and *gyrA* but two for *rpoS* genes. These results indicated a low degree of diversity within the studied species. This low variability among *E. amylovora* strains was observed previously at the phenotypic and genetic levels (Dye 1968; Brenner et al. 1974; Vantomme et al. 1982; Verdonck et al. 1987; Zhang and Geider 1997; Kim et al. 1999, 2001).

Application of *rec*A PCR–RFLP allowed the discrimination of *E. amylovora* and *E. pyrifoliae* strains and differentiation of *E. amylovora* strains originating from Canada. The *E. amylovora* strains were placed into two RFLP groups. The strains EaP-1, EaKp1/00 and EaRUB7, originating from Kentville/Nova Scotia in Canada, were separated from the other 16 *E. amylovora* strains. The diversity of strains from this region was observed earlier when PFGE and SSR analyses were performed (Jock and Geider 2004; Jock et al. 2003).

Few molecular techniques such as RAPD (Momol et al. 1997), PFGE (Jock et al. 2002; Jock and Geider 2004), and SSR analysis (Ruppitsch et al. 2004) can differentiate *E. amylovora* strains. PFGE patterns seem to be quite stable over time, whereas SSRs may change quickly in a population of field strains. Random primers can produce variable patterns, which can even be dependent on DNA preparation. Based on dominant bands, ribotyping was described to differentiate strains isolated from *Maloidea* and *Rubus* hosts (McManus and Jones 1995). A lack of RFLP patterns related to these host plants or year of isolation is in agreement with similar biochemical, physiological, serological or genetic characteristics of a series of Irish strains (Brennan et al. 2002). Higher variability was observed among strains from North America, which is considered the origin of the pathogen, than among strains from Europe, where the disease has been present only for 50 years (Jock and Geider 2004).

In the case of *Erwinia* spp. strains originating from Japan, some heterogeneity was observed in SSR analysis (Jock et al. 2003), in *gyrB* and *rpoD* (Matsura et al. 2007) and *hrpN* gene sequences, and in PFGE assays (Jock and Geider 2004). Six tested strains originating from Japan can be divided into three groups according to RFLP analysis of three genes: *recA, gyrA* and *rpoS*. *Erwinia* spp. strain Ejp556 indicated the highest similarity to *E. pyrifoliae* strains. A close relationship between

this strain and *E. pyrifoliae* was observed earlier, when a sequence of the *cps* region was analyzed (Kim et al. 2001). *Erwinia* spp. strains Ejp546, Ejp547, Ejp557 and Ejp562 shared almost the same genotype in the *recA* and *rpoS* polymorphism analysis (Fig. 4). There was no amplification product of the *gyrA* gene fragment when genomic DNA of these strains was used as a target. Strain Ejp617 differed in the *recA* gene sequence from other *Erwinia* spp. strains (Fig. 1). These observations confirmed earlier studies of 16S rRNA, *cps* and *hrpN* genes (Kim et al. 2001; Jock and Geider 2004).

The polymorphism of the three housekeeping genes *recA*, *gyrA* and *rpoS* amplified from genomic DNA of *Erwinia* strains was in agreement with the results of several molecular methods described by Jock and Geider (2004) and confirmed that *Erwinia* spp. strains isolated in Japan are more related to *E. pyrifoliae* than to *E. amylovora*.

The described method is useful for rapid identification and differentiation of the investigated species without nucleotide sequencing. The whole procedure, including genomic DNA isolation, lasted 48 hours. The procedure is even shorter when whole bacterial cells are used for the PCR reaction (preferentially after heat lysis in Tween 20) and should be applied when there is an indication that bacteria from *E. amylovora* or *E. pyrifoliae* may cause a necrotic disease in host plants. Due to the RFLP profiles obtained and also considering the cost of restriction endonucleases, we recommend the amplification of *recA* and *rpoS* gene fragments and their digestion with *Hin*6I for quick and effective differentiation of both species (Figs. 1b and 3b).

PCR-RFLP for the conservative housekeeping genes seems to be a simple and accurate method for identification and differentiation of the strains from the species *E. amylovora* and other *Erwinia* species.

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