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ORIGINAL ARTICLE

Characterization of *Erwinia amylovora* strains from different host plants using repetitive-sequences PCR analysis, and restriction fragment length polymorphism and short-sequence DNA repeats of plasmid pEA29

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Abstract

Aims: The three main aims of the study were the assessment of the genetic relationship between a deviating *Erwinia amylovora* strain isolated from *Amelanchier* sp. (Maloideae) grown in Canada and other strains from Maloideae and Rosoideae, the investigation of the variability of the *Pst*I fragment of the pEA29 plasmid using restriction fragment length polymorphism (RFLP) analysis and the determination of the number of short-sequence DNA repeats (SSR) by DNA sequence analysis in representative strains.

Methods and Results: Ninety-three strains obtained from 12 plant genera and different geographical locations were examined by repetitive-sequences PCR using Enterobacterial Repetitive Intergenic Consensus, BOX and Repetitive Extragenic Palindromic primer sets. Upon the unweighted pair group method with arithmetic mean analysis, a deviating strain from *Amelanchier* sp. was analysed using amplified ribosomal DNA restriction analysis (ARDRA) analysis and the sequencing of the 16S rDNA gene. This strain showed 99% similarity to other *E. amylovora* strains in the 16S gene and the same banding pattern with ARDRA. The RFLP analysis of pEA29 plasmid using *Msp*I and *Sau*3A restriction enzymes showed a higher variability than that previously observed and no clear-cut grouping of the strains was possible. The number of SSR units reiterated two to 12 times. The strains obtained from pear orchards showing for the first time symptoms of fire blight had a low number of SSR units.

Conclusions: The strains from Maloideae exhibit a wider genetic variability than previously thought. The RFLP analysis of a fragment of the pEA29 plasmid would not seem a reliable method for typing *E. amylovora* strains. A low number of SSR units was observed with first epidemics of fire blight.

Significance and Impact of the Study: The current detection techniques are mainly based on the genetic similarities observed within the strains from the cultivated tree-fruit crops. For a more reliable detection of the fire blight pathogen also in wild and ornamentals Rosaceous plants the genetic features of deviating *E. amylovora* strains have to be studied in detail.

Introduction

Erwinia amylovora (Burrill) Winslow *et al.* causes one of the most severe diseases affecting the Rosaceae family worldwide. Plant species, especially those belonging to Maloideae and Rosoideae subfamilies, can be infected by the bacterium. Plant quarantine measures may slow but often fail to prevent the eventual introduction into new areas. Assessing the genetic variability of pathogens is a fundamental prerequisite to develop effective diagnostic

protocols. Recently, several molecular techniques have revealed unexpected genetic variability among *E. amylovora* strains. Both repetitive-sequence PCR (rep-PCR) and random amplified polymorphic DNA (RAPD) analysis allowed the differentiation of strains isolated from Maloideae plant species such as pear (*Pyrus communis* L.), apple (*Malus domestica* Borkh.), quince (*Cydonia oblonga* L.), hawthorn (*Crataegus* spp.) from the strains obtained from Rosoideae such as raspberry (*Rubus* spp.) (McManus and Jones 1995a; Momol *et al.* 1997). Differentiation of *Rubus* strains was also possible using 23S rDNA sequence analysis (Maes *et al.* 1996), DNA restriction enzyme analysis of the 16S–23S intergenic spacer regions (Momol *et al.* 1999) and amplified fragment length polymorphism (AFLP) analysis (Rico *et al.* 2004). In addition, relationship between some *E. amylovora* strains and their geographical origins has been inferred using pulsed-field gel electrophoresis (Zhang and Geider 1997; Jock *et al.* 2002).

An important feature of the bacterium utilized both for characterization and detection is plasmid pEA29. With few exceptions, all *E. amylovora* strains carry a similar plasmid of *c.* 29 kb that can be detected by PCR amplification of 900 bp *Pst*I fragment (Bereswill *et al.* 1992). However, the *Pst*I fragment of some strains was larger than the expected size (McManus and Jones 1995b). Moreover, variability in the length of the DNA fragment was observed after its digestion with *Msp*I and *Sau*3A restriction enzymes, and strains were classified into three groups according to the length of the PCR products (Lecomte *et al.* 1997). The fragment length variability is due to the presence of different numbers of short-sequence DNA repeats (SSR) (Schnabel and Jones 1998). The number of SSR units has been recently used to try to type *E. amylovora* strains (Kim and Geider 1999; Jock *et al.* 2003; Ruppitsch *et al.* 2004), and, in case of a very low or very high number of SSR repeats the traceability of certain strains was possible (Ruppitsch *et al.* 2004).

Recently, an *E. amylovora* strain isolated from *Amelanchier* sp. (Maloideae) grown in Canada, showed a restricted pathogenicity upon artificial inoculation to apple, pear, raspberry and *Amelanchier* sp. plants. In fact, only the latter plant showed an extensive progression of the lesion along the twig (Giorgi and Scortichini 2005). In addition, also the assessment of the *hrpN* and *dspA/E* genes involved in the pathogenicity of *E. amylovora* pointed out some missense point mutations that could have implications for the particular pathogenicity of the strain (Giorgi and Scortichini 2005). Consequently, further assessment to verify the genetic relationship of this strain with representative strains of *E. amylovora* was undertaken. In the present study, after the screening of the genetic variability of 93 *E. amylovora* strains obtained from different host plants belonging to 12 different genera and inferred using rep-PCR, it was observed that the strain from *Amelanchier* sp. and three strains isolated from *Rubus* spp. had a different genomic fingerprint.

The objectives of this study were to: (i) establish the genetic relationship between the strain isolated from *Amelanchier* sp. (Maloideae) and the other strains from Maloideae and Rosoideae using amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA gene sequencing; (ii) assess the collection using restriction fragment length polymorphism (RFLP) analysis for checking the variability in length of the *Pst*I fragment of pEA29 plasmid and to verify the reliability of strain grouping with this method; (iii) further analyse the occurrence and the number of SSR units in *E. amylovora* from different host plants and to check the number of SSR units in 32 strains obtained from two pear orchards showing for the first time symptoms of fire blight.

Materials and methods

Bacterial strains and growth medium

Erwinia amylovora strains used in this study are listed in Table 1. The strains marked with Istituto Sperimentale per la Frutticoltura (ISF) were isolated for this study from diseased pear specimens, all the others were obtained from international or national culture collection (Table 1). Pear twigs or fruits showing symptoms of fire blight were used for isolation. Fragments of tissue at the margin of lesion were crushed in sterile mortars containing 5 ml of sterile saline (SS) (0·85% of NaCl in distilled water). Ten-fold serial dilutions in tubes were performed. Subsequently, aliquots of 0·1 ml were spread on to Petri dishes containing nutrient sucrose agar (NSA) [28·0 g of nutrient agar (Oxoid, Basingstoke, UK) supplemented with 50·0 g of sucrose, per litre]. The plates were incubated at 25–27°C for 3 days. With the levaniform, whitish colonies suspected to belong to *E. amylovora*, confirmatory tests (i.e. tobacco hypersensitivity, absence of fluorescent pigments on the medium B of King *et al.* (1954) (KB), induction of necrosis and oozing in immature pear fruits, SDS-PAGE of whole-cell protein extracts and comparison with *E. amylovora* type-strain NCPPB 683 and other representative strains previously isolated in Italy was carried out. The isolates inducing the hypersensitivity reaction in tobacco leaves, negative for the fluorescent pigments production on KB, inciting necrosis and oozing on immature pear fruits and showing the same protein profile as *E. amylovora* NCPPB 683 and the other representative Italian strains, were retained to belong to *E. amylovora*. For this study, all the 93 strains used were routinely grown on NSA, at 25–27°C.

Table 1 *Erwinia amylovora* strains used in this study and number of single-sequence DNA repeats (SSR)

Strain	Host plant/cultivar	Origin	Year of isolation	SSR
BPIC 845*	*Pyrus communis*	Greece	1984	
BPIC 847	*P. communis*/P. Crassane	Greece	1984	
BPIC 913	*P. communis*/De Cayan	Greece	1985	
BPIC 918*	*Pyrus amygdalifoliae*	Greece	1986	6
BPIC 930	*Malus domestica*	Greece	1986	4
BPIC 974	*P. communis*	Greece	1987	9
BPIC 1093*	*P. communis*	Greece	1990	9
BPIC 1625	*P. communis*	Greece	1991	
H-895*	*M. domestica*/G. Delicious	Hungary	1996	11
H-902	*Cydonia oblonga*	Hungary	1996	7
ICMP 13413	*P. communis*	Hungary	1996	
ICMP 13414*	*Sorbus aria*	Hungary	1996	6
ICMP 13415	*M. domestica*/Johnatan	Hungary	1996	10
ICMP 13417	*Mespilus germanica*	Hungary	1996	
IPV-BO 2956	*P. communis*	Italy	1996	
ISF Ab 1*	*P. communis*/Abate	Italy	2001	5
ISF Ab 2	*P. communis*/Abate	Italy	2001	5
ISF Ab 3*	*P. communis*/Abate	Italy	2001	5
ISF Ab 2-1	*P. communis*/Abate	Italy	2002	5
ISF Ab 2-2	*P. communis*/Abate	Italy	2002	5
ISF Ab 2-3	*P. communis*/Abate	Italy	2002	5
ISF Ab 2-4	*P. communis*/Abate	Italy	2002	4
ISF Ab 2-5	*P. communis*/Abate	Italy	2002	4
ISF Ab 2-6	*P. communis*/Abate	Italy	2002	4
ISF Ab 2-7	*P. communis*/Abate	Italy	2002	5
ISF Ab 2-8	*P. communis*/Abate	Italy	2002	5
ISF Ab 2-9	*P. communis*/Abate	Italy	2002	5
ISF Conf 1*	*P. communis*/Conference	Italy	2001	5
ISF Conf 2	*P. communis*/Conference	Italy	2001	5
ISF Cot 1*	*C. oblonga* A	Italy	2001	5
ISF-CotP 1	*Cotoneaster salicifolia*	Italy	2003	
ISF-CotP 2	*C. salicifolia*	Italy	2003	
ISF-CotP 3	*C. salicifolia*	Italy	2003	
ISF-CotP 4	*C. salicifolia*	Italy	2003	
ISF-Ea 1/79 Rif	*P. communis*	USA	Unknown	
ISF-Joinee*	*P. communis*	USA	Unknown	
ISF SM 1	*P. communis*/S. Maria	Italy	2001	5
ISF SM 2	*P. communis*/S. Maria	Italy	2001	5
ISF SM 3	*P. communis*/S. Maria	Italy	2001	5
ISF SM 2-1	*P. communis*/S. Maria	Italy	2002	6
ISF SM 2-2*	*P. communis*/S. Maria	Italy	2002	6
ISF SM 2-3*	*P. communis*/S. Maria	Italy	2002	6
ISF SM-Fr 1*†	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 2*	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 3*	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 4*	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 5*†	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 6*†	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 7*	*P. communis*/S. Maria	Italy	2002	4
ISF SM-Fr 8*	*P. communis*/S. Maria	Italy	2002	4
ISF-WC31	*P. communis*	USA	Unknown	2
ISF Will 1*	*P. communis*/William	Italy	2001	5
ISF Will 2	*P. communis*/William	Italy	2001	5
ISF Will 3	*P. communis*/William	Italy	2001	5
IVIA 198-11*	*Crataegus* sp.	Spain	1996	
IVIA 1525-1*	*Cotoneaster* sp.	Spain	1996	
IVIA 1767-3*	*Malus* sp.	Spain	1997	6

Table 1 Continued

Strain	Host plant/cultivar	Origin	Year of isolation	SSR
IVIA 1892-1	*P. communis*	Spain	1998	
IVIA 1951-2*	*Pyracantha* sp.	Spain	1998	4
NCPPB 595	*P. communis*	UK	1958	
NCPPB 683*‡	*P. communis*	UK	1959	5
NCPPB 2080*	*P. communis*	New Zealand	1955	5
NCPPB 2292*	*Rubus idaeus*	USA	1949	
NCPPB 2293*	*R. idaeus*	USA	1949	3
NCPPB 3159*	*M. domestica*	Italy	1981	5
PD 103*	*Rubus* sp.	USA	1978	0
PD 394*	*M. domestica*/Mantet	the Netherlands	1983	
PD 2233*	*C. oblonga*	Bulgaria	1993	
PD 2912*	*Eriobotrya japonica*	Israel	1997	9
PD 2913*	*C. oblonga*	USA	Unknown	
PD 2914*	*Dichomanthes* sp.	UK	1996	
PD 2915*	*Amelanchier* sp.	Canada	1996	7
PD 3368*	*M. domestica*	the Netherlands	1998	12
PD 3678*	*Crataegus monogyna*	Germany	1999	
PD 3891*	*P. communis*/Wildeman	the Netherlands	2000	4
PD 4071*	*Sorbus aucuparia*	Germany	1999	
UniBa-Al 11	*P. communis*	Albania	1995	5
UniBa-Al 12	*P. communis*	Albania	1995	6
UniBa-BA5	*P. communis*	Italy	1994	
UniBaBA6*	*Cotoneaster* sp.	Italy	1995	8
UniBa-BA7	*P. communis*	Italy	1997	
UniBa-BR1	*P. communis*	Italy	1990	
UniBa-LE1	*P. communis*	Italy	1990	4
UniBa-LE2	*P. communis*	Italy	1990	
UniBa-LE4	*P. communis*	Italy	1992	
UniBa-LE8	*P. communis*	Italy	2001	5
UniBaLE9*	*M. domestica*	Italy	2001	
UniBaLE10*	*Cotoneaster* sp.	Italy	2001	5
UniCt-Sic1	*P. communis*	Italy	1991	4
UniCt-Sic2	*P. communis*	Italy	1991	4
28CS*	*P. communis*	Czech Republic	1990	
85CS	*P. communis*	Czech Republic	1990	
90CS	*P. communis*	Czech Republic	1990	

BPIC, Benaki Phytopathological Institute Collection, Kiphissia-Athens, Greece; CS, Collection of the Institute of Plant Molecular Biology, Ceske Budjovice, Czech Republic; H, Collection of the Plant Health and Soil Conservation Service, Pecs, Hungary; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; IPV-BO, Culture Collection of the University of Bologna, Italy; ISF, Culture Collection of the C.R.A.-Istituto Sperimentale per la Frutticoltura, Roma, Italy; IVIA, Collection of Instituto Valenciano de Investigaciones Agraria, Moncada-Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; PD, Culture Collection of Plant Protection Service, Wageningen, the Netherlands; UniBa, Culture Collection of the University of Bari, Bari, Italy; UniCt, Culture Collection of the University of Catania, Catania, Italy.

*Strains assessed with amplified ribosomal DNA restriction analysis.

†Strains isolated from diseased fruits.

‡*Erwinia amylovora* type strain.

DNA preparation

To prepare total genomic DNA, a modification of the technique used by Smith *et al.* (1995) was used. For each strain, a loop of bacteria (*c.* 3-mm diameter) of a single colony that had been grown for 24 h on NSA at 25–27°C, was suspended in SS, and centrifuged at 12 000 ***g*** for 2 min. The supernatant was discarded and the pellet was suspended in bidistilled, filtered, sterilized water up to an optical density corresponding to $1–2 \times 10^8$ CFU ml^{-1}. The suspension was placed in boiling water for 10 min and then stored at −20°C.

Repetitive sequences PCR

Genomic fingerprints of all *E. amylovora* strains listed in Table 1 were assessed by rep-PCR using Enterobacterial Repetitive Intergenic Consensus (ERIC), Repetitive Extragenic Palindromic (REP) and BOX-A1R subunit of the BOX element of *Streptococcus pneumoniae* (BOX) primer sets. The PCR method used was that of Louws *et al.* (1994). The primer sets were synthesized by Eurogentec (Seraing, Belgium). Amplification was performed in a Bio-Rad Gene Cycler™, version 1.5. (Bio-Rad, Hercules, CA, USA). Each 30 μl of reaction mixture contained 1X PCR buffer (10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl and 0·1% Triton X-100, pH 9·0), 200 μmol l^{-1} each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 2 mmol l^{-1} $MgCl_2$, 60 pmol of each primer, 1·0 U of *Taq* polymerase (Promega), and 4 μl of lysed cell suspension. A negative control, consisting of the same reaction mixture but without DNA template, was included in each amplification procedure. The thermal cycling procedure was that used by Louws *et al.* (1994). Subsequently, the PCR amplification products were separated by gel electrophoresis on a 2·5% agarose (Seakem-Cambrex, Rockland, ME, USA) gel in 1X Tris-borate–EDTA (TBE) buffer (80 mmol l^{-1} Tris-borate, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA; pH 8·0) at 60 V cm^{-1} for 3 h, stained with ethidium bromide, visualized with a UV transilluminator, and photographed with Polaroid type 55 film (Polaroid, Cambridge, MA, USA). The PCR amplifications were performed in duplicate. The method of Smith *et al.* (1995) was used for analysis. The clearly resolved bands present in both amplification gels were scored and recorded to build up a binary matrix. Similarity coefficients for all pairwise combinations were determined by using the Dice (1945)'s coefficients and were clustered by unweighted pair group method with arithmetic means (UPGMA), using the NTSYS-pc software (Exeter Software, New York, NY, USA), version 2.11j (Rohlf 2000). Phenograms were constructed with the tree display option (TREE). A cophenetic value matrix was calculated using the COPH option and compared with the original similarity matrix using the MXCOMP option to test the goodness-of-fit of the cluster analysis.

ARDRA analysis

The basic ARDRA technique described by Vaneechoutte *et al.* (1992) was utilized. The DNAs coding for the 16S rRNAs of 40 *E. amylovora* strains (see Table 1) were amplified with primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′). These primers were designed by Grifoni *et al.* (1995) on the basis of the conserved bacterial sequences at the 5′- and 3′-ends of the 16S rRNA gene and allowed the amplification of almost the entire gene. Each 50-μl (final volume) of PCR mixture contained 6 μl of lysed cells suspension, Promega *Taq* buffer (1·5 mmol l^{-1} $MgCl_2$), each deoxynucleoside triphosphate at a concentration of 200 μmol l^{-1}, 36 pmol of each primer and 1·5 U of *Taq* DNA polymerase (Promega). The reaction mixtures were incubated in a Bio-Rad Gene Cycler™, version 1·5 at 95°C for 2 min and then subjected to 35 cycles at 95°C for 30 s, 30 s of annealing (60°C for the first five cycles, 55°C for the next five cycles, and 50°C for the last 25 cycles), and 72°C for 4 min. Finally, the mixtures were incubated at 72°C for 10 min and then at 60°C for 10 min. Five microlitres of each amplification mixture was analysed by 1·5% agarose (Seakem) gel electrophoresis in 1X TBE buffer containing 0·5 μg of ethidium bromide per ml at 5·0 V cm^{-1}. A volume of 15 μl aliquot of each PCR mixture containing *c.* 1·5 μg of amplified 16S rDNA was digested with *Hae*III and *Hin*fI restriction endonucleases as recommended by the manufacturer (Promega). The reaction products were analysed by 2·5% agarose (Seakem) gel electrophoresis in 1X TBE buffer containing 0·5 μg of ethidium bromide per ml. Other Enterobacteriaceae, namely *Erwinia pyrifoliae* CFBP 4172 (provided by C. Manceau, INRA, France) and *Brenneria nigrifluens* NCPPB 2020 were used for comparative purposes.

16S rDNA sequence analysis

The 16S rDNA sequences of *E. amylovora* PD 103 and PD 2915 were obtained by amplifying and sequencing three overlapping fragments of 490, 598 and 562 bp with three primer sets: fD2(5′-AGAGTTTGATCATGGCTCAG-3′) and 16SA reverse (5′-GTAACGTCAATGCAACAGGTT-3′); 16SB forward (5′-TTGTAAAGTACTTTCAGCGGG-3′) and 16SB reverse (5′-TTCCGCATCTCTGCAGAATT-3′); 16SC forward (5′-GTGGTTTAATTCGATGCAACG-3′) and rP1 (5′-ACGGTTACCTTGTTACGACTT-3′). The rDNA PCRs were carried out in a total volume of 30 μl containing (final concentrations) 1X PCR buffer (10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl, and 0·1% Triton X-100, pH 9), 50 pmol of each primer, 1·0 U *Taq* DNA polymerase (Promega), 0·2 mmol l^{-1} each of dATP, dCTP, dGTP and dTTP (Promega), 1·6 mmol l^{-1} $MgCl_2$, and 4 μl of lysed cell suspension. The reaction mixtures were incubated in a Bio-Rad Gene Cycler™, version 1·5 at 95°C for 7 min followed by 35 cycles of 94°C for 1 min, 43°C for 1 min and 72°C for 2 min and a final incubation step at 72°C for 5 min. Prior to sequencing PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. All the sequences

were determined at MWG-Biotech AG (Ebersberg, Germany) directly from the purified fragments, using the corresponding amplification primers. Sequences were aligned for the same strain. The relatedness of 16S rDNA sequences of the tested strains with those of some *E. amylovora* strains obtained from the GenBank accession numbers were determined with the PC/Gene program CLUSTAL W MULTIPLE SEQUENCE ALIGNMENTS (version 1.82) (Thompson *et al.* 1994), using the cladogram tree of the neighbour-joining (NJ) options.

RFLP analysis of plasmid pEA29

The procedures described by Lecomte *et al.* (1997) were slightly modified as follows. Primers were synthesized by Eurogentec according to the sequences provided by Bereswill *et al.* (1992): primer A (5′-CGGTTTTTAACGCTGGG-3′); primer B (5′-CGGCAAATACTCGGATT-3′). The final PCR mixture (50 μl) contained 20 mmol l^{-1} of Tris–HCl (pH 8·0), 100 mmol l^{-1} of KCl, 0·1 mmol l^{-1} of EDTA, 1 mmol l^{-1} of DTT, 0·5% of Tween 20, 2 mmol l^{-1} of $MgCl_2$, 250 μmol l^{-1} of each dATP, dCTP, dGTP, dTTP (Promega), 0·5 U of *Taq* DNA polymerase (Promega) and 50 pmol of each primer. PCR amplification was performed in a Bio-Rad GenCycler™, version 1.5 and included a denaturation step at 92°C for 1 min, followed by 35 cycles at 92°C for 1 min, 52°C for 1 min and 72°C for 1 min with a final DNA extension step at 72°C for 2 min. Aliquots of 10 μl of the PCR amplification were analysed on 1% agarose gel (Seakem) for 30 min at 100 V cm^{-1}, in Tris-acetate buffer. A 100-bp DNA ladder (Promega) was used as a size marker. For the RFLP analysis, aliquots of 40 μl of PCR products were purified by ethanol precipitation. The purified DNAs were resuspended in 16 μl of sterile water. Eight microlitres of aliquots were used for digestion with 5 U *Msp*I and 5 U *Sau*3A restriction enzymes (Promega) for 2 h in a total of 15-μl volume at 37°C. The samples were analysed on 3·0% agarose gel (Seakem).

Short-sequence DNA repeats analysis

The SSRs analysis was performed for 61 *E. amylovora* strains. For detecting the presence of SSR units, 10 μl of aliquots of the pEA29 PCR fragment were amplified with primer RS1 (5′-ACCTCAGTGCGATTACAG-3′) and RS2c (5′-GTCCCATTCTGTGTTAAG-3′) (Kim and Geider 1999). The products were analysed on 8% polyacrylamide gel. The number of SSR units was established following the procedure described by Ruppitsch *et al.* (2004). Briefly, the A/B PCR fragment was amplified by the use of primer sB (5′-TGTAAAACGACGGCCAGTGGGCAAATACTCGGATT-3′) and primer sA (5′-CAGGAAACAGCTATGACCCGGTTTTTAACGCTGGG-3′) (MWG-Biotech) containing the M13 recognition sequence for subsequent DNA sequencing analysis. The amplified DNA fragment was ethanol precipitated and sequence analysis was performed by cycle sequencing (SequiTherm Excel II Cycle Sequencing kit; Epicentre, Madison, WI, USA) with fluorescent-labelled primers M13 univ. (5′-TGTAAAACGACGGCCAGT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACC-3′) (MWG-Biotech) using an automated DNA sequencer Licor 4200S (LI-COR, Lincoln, NE, USA) according to the manufacturer's instructions.

Results

Repetitive sequences PCR analysis

The genetic relationship within *E. amylovora* strains was assessed using rep- PCR and ERIC, REP and BOX primer sets. Reproducible DNA fingerprints were generated from total DNA of all strains listed in Table 1. REP and ERIC primers resulted more discriminative than BOX in pointing out variability within *E. amylovora* strains and are discussed herein. A cophenetic value of >0·91 and 0·92 was determined for the two similarity matrix, respectively, indicating a high goodness-of-fit for the cluster analysis. UPGMA analysis was performed by combining the data obtained from REP-PCR and ERIC-PCR. A total of 24 clearly resolved bands were selected for composing the binary matrix. Representative genomic fingerprints are shown in Fig. 1 and the corresponding dendrogram is shown in Fig. 2. The majority (89 of 93) of *E. amylovora* strains showed the same DNA fingerprint profile. Diversity was ascertained for four strains, namely PD 2915, isolated from *Amelanchier* sp. and NCPPB 2292, NCPPB 2293 and PD 103, isolated from *Rubus* spp. *E. amylovora* PD 2915 clustered separately from the strains isolated from other Maloideae plant species. Its DNA fingerprint profile was more similar to the profiles showed by NCPPB 2292 and 2293, isolated from *Rubus* spp. By contrast PD 103, isolated from *Rubus* sp., resulted more similar to the profile showed by the strains obtained from Maloideae. ERIC-PCR allowed the differentiation of NCPPB 2292 and NCPPB 2293.

ARDRA

To ascertain if ARDRA analysis also revealed the presence of different groups, with a subset of 40 representative *E. amylovora* strains (Table 1) including all strains from *Rubus* spp. and *Amelanchier* sp., the 16S rDNA was amplified and digested with *Hin*fI and *Hae*III restriction endonucleases. Only *Hae*III yielded a restriction pattern differentiating *E. amylovora* within the genus *Erwinia* (Bereswill *et al.* 1995). Using this endonuclease, fragments

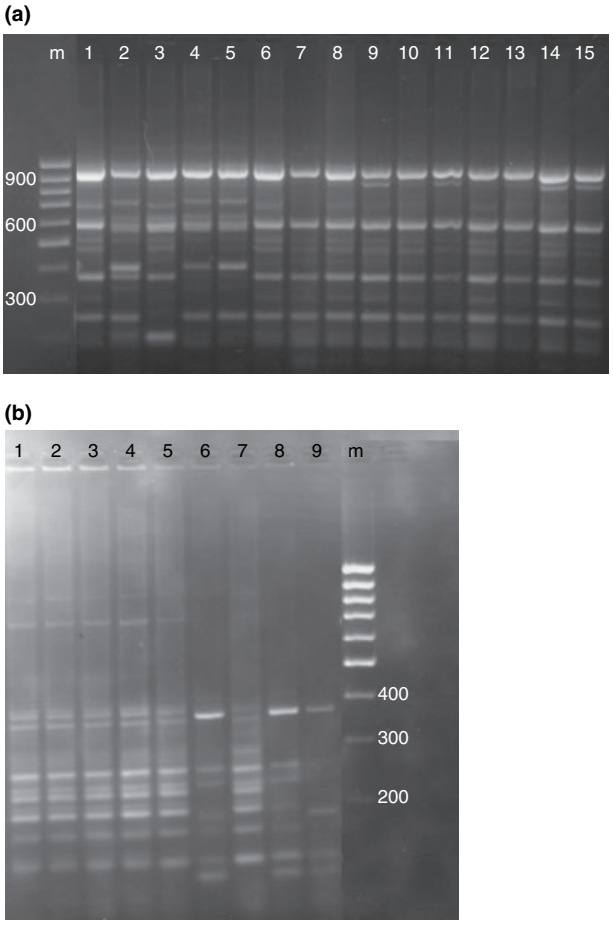

Figure 1 Repetitive-sequence PCR fingerprint patterns of genomic DNA from *Erwinia amylovora* strains obtained by using Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC) primer sets. M, molecular size marker (100-bp DNA ladder; Gibco-BRL; Gibco-Invitrogen, Carlsbad, CA, USA). (a) REP: lane 1, *E. amylovora* NCPPB 683; lane 2, PD 2915; lane 3, PD 103; lane 4, NCPPB 2292; lane 5, NCPPB 2293; lane 6, PD 2913; lane 7, PD 2233; lane 8, PD 2912; lane 9, PD 2914; lane 10, PD 3678; lane 11, IVIA 198.11; lane 12, PD 4071; lane 13, PD 3368; lane 14, PD 3891; lane 15, IVIA 1892.1. (b) ERIC: lane 1, *E. amylovora* ISF CotP 1; lane 2, ISP CotP 2; lane 3, ISP CotP 3; lane 4, ISP CotP 4; lane 5, NCPPB 683; lane 6, PD 2915; lane 7, PD 103; lane 8, NCPPB 2292; lane 9, NCPPB 2293.

of 204, 222 and 317 bp and a triplet containing fragments of 164, 165 and 170 bp were observed for all the 40 *E. amylovora* strains, and no grouping was possible. When compared with *E. amylovora*, *E. pyrifoliae* and *B. nigrifluens*, used as outgroup, gave rise to a clearly different banding patterns (Fig. 3).

16S rDNA sequencing and comparison of strains

The 16S rDNA sequences of *E. amylovora* strains PD 103 and PD 2915, showing a different genomic pattern upon rep-PCR, were sequenced, and the 1506 nucleotides were compared by means of Clustal W algorithm and NJ clustering method with the corresponding sequences of *E. amylovora* BC199 and BC201, isolated from *Rubus* spp. (GenBank accession numbers AF141891 and AF141892 respectively), BC 204, isolated from *Rubus fruticosus* (GenBank accession number AF141895), Ea321 and NCPPB 683, isolated from *P. communis* (GenBank accession numbers AF140337 and AF140341 respectively). The resulting cladogram is shown in Fig. 4. The *E. amylovora* strains isolated from *P. communis* clustered separately. Interestingly, *E. amylovora* PD 2915, obtained from *Amelanchier* sp., was grouped with two strains isolated from *Rubus* spp.

Sequence accession numbers

The following nucleotide sequences were deposited in the EMBL GenBank nucleotide sequence database: 16S rDNA gene from *E. amylovora* PD 2915 and *E. amylovora* PD 103 (accession numbers AJ746201 and AJ746202 respectively).

RFLP analysis of plasmid pEA29 and SSR

With the sole exception of PD 103, isolated from *Rubus* sp., both *Msp*I and *Sau*3A enzymes cleaved the amplified fragment of the plasmid pEA29 in all 93 *E. amylovora* strains tested. After the digestion with *Msp*I we found a greater variability in the larger fragment than expected according to the results of Lecomte *et al.* (1997). This was even more evident using *Sau*3A restriction enzyme. In fact, besides a rather stable fragment of *c.* 180 bp, we found a great variability in the discriminative fragments between 200 and 400 bp. We found a sort of continuous variability in the larger fragment starting from around 250 bp of PD 2913 to 280–290 bp of PD 2915 (Fig. 5). For this reason any clear-cut grouping of the strains was pointed out, although, tentatively eight patterns, from A to H were scored (Fig. 5).

The PCR amplification of a fragment of plasmid pEA29 using the primers RS1 and RS2c, followed by the electrophoresis on 8% polyacrylamide gel allowed the preliminary detection of the SSR units (Fig. 6). The number of SSR units, using the frame GAATTACA, was subsequently determined for 61 *E. amylovora* strains, including 32 strains obtained from two pear orchards in Italy that showed for the first time symptoms of fire blight. The number of SSR units is reported in Table 1. The motif of eight nucleotides reiterated from two to 12 times within the *Pst*I fragment of the pEA29 plasmid of *E. amylovora*. The minimum of two SSR units ever found was detected only in strain ISF-WC31 from the USA. The majority of the Italian strains had an SSR number from four to six

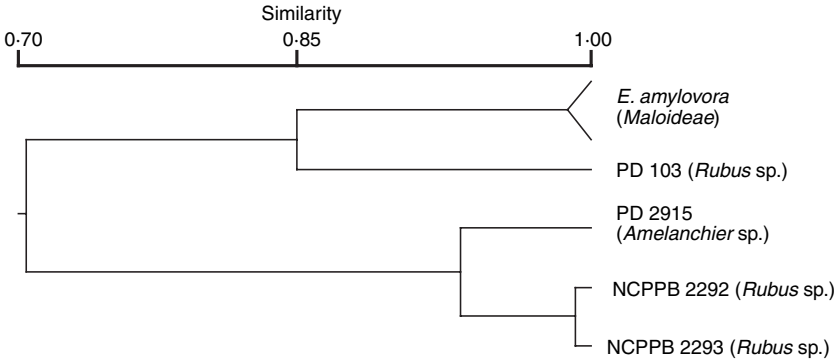

Figure 2 Dendrogram of genetic relatedness of the combined repetitive-sequence PCR data inferred using Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergenic Consensus primer sets and generated by 93 *Erwinia amylovora* strains. Unweighted pair group method with arithmetic mean analysis was performed using Dice's coefficients. The scale indicates the degree of genetic relatedness between strains. From the 93 *E. amylovora* strains tested, 89 showed the same pattern. Four strains showed distinct banding patterns.

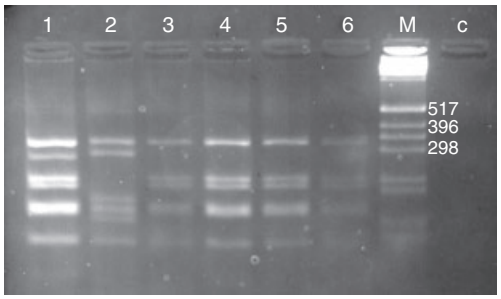

Figure 3 Amplified ribosomal DNA restriction analysis banding patterns for amplified 16S rDNA obtained by using *Hae*III restriction endonuclease for *Erwinia amylovora*, *Erwinia pyrifoliae* and *Brenneria rubrifaciens* strains. M, molecular size marker (1-kb DNA ladder; Gibco-BRL). Lane 1, *E. pyrifoliae* CFBP 4172; lane 2, *B. rubrifaciens* NCPPB 2020; lane 3, *E. amylovora* PD 2915; lane 4, *E. amylovora* UniCt Sic 1; lane 5, *E. amylovora* IVIA 1951-2; lane 6, *E. amylovora* IVIA 1767-3; C, negative control.

units. The number of SSR units of the strains isolated from the pear orchards with first symptoms of fire blight was low and varied from four to six. All the eight strains isolated from an infected pear fruit (ISF SM-Fr from 1 to 8) possessed four SSR units. Nine SSR units were found in two strains from Greece. Strains with 10 or 11 SSR units were from Hungary. No SSR units were found in strain PD 103 from *Rubus* sp. Strain PD 2915 from *Amelanchier* sp. contains seven SSR units. No other correlation was pointed out between the number of SSR units and particular features of the strains tested.

Discussion

This study clearly showed that, upon rep-PCR, an *E. amylovora* strain, PD 2915, isolated from an *Amelanchier* sp. (Maloideae) grown in Canada, diverges from all the 89 strains obtained from the other Maloideae plant species. In addition, PD 2915 clustered separately from all other *E. amylovora* strains by 16S rDNA gene sequence comparison. However, such a diversity is most probably, within the range of variability of a species. In fact, ARDRA analysis, performed with *Hae*III endonuclease, which allows species differentiation within the genus *Erwinia*, did not reveal a different restriction pattern. The sequence homology with other *E. amylovora* strains from *P. communis* was 99%. The difference of this strain was also pointed out by pathogenicity tests and by assessing and comparing genes involved in the pathogenicity of *E. amylovora* such as *hrpN* and *dspA/E* (Giorgi and Scortichini 2005). By using AFLP, Rico *et al.* (2004) also found a deviating strain isolated from *Crataegus* sp. in the USA that showed around 62% of dissimilarity with other *E. amylovora* strains from Maloideae. This confirms once more that a greater genetic diversity of *E. amylovora* has to be expected from strains isolated in North America, the centre of origin of the pathogen (McManus and Jones 1995a).

The assessment of more *E. amylovora* strains obtained from wild and ornamental Rosaceous plants using different techniques (PCR-based molecular fingerprinting, presence and characterization of the pEA29 plasmid, gene sequencing) may improve the detection of the pathogen in asymptomatic plants. In fact, nowadays the detection of the pathogen is mainly based on the features shown by the strains obtained from cultivated crops. The creation of an international databank concerning *E. amylovora* features would greatly help the detection tasks.

By using rep-PCR, similar results were obtained by McManus and Jones (1995a). Also in their study all the strains from *Rubus* spp. (Rosoideae) clustered separately from the strains from Maloideae, even if ARDRA analysis carried out with *Hae*III did not reveal any diversity in the

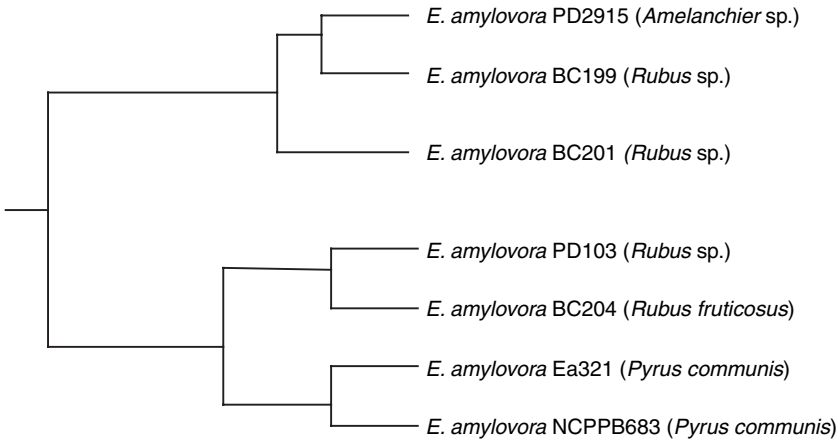

Figure 4 Cladogram based on 16S rDNA nucleotide sequence of *Erwinia amylovora* strains from *Amelanchier* sp., *Rubus* spp. and *Pyrus communis* inferred by means of Clustal W multiple sequence alignment. The branch lengths are not proportionate to sequence divergence. Neighbour joining was chosen as clustering algorithm.

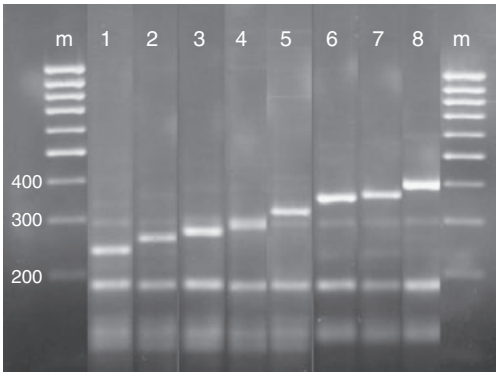

Figure 5 Representative cleavage patterns of the PCR amplification products from plasmid pEA29 of *Erwinia amylovora* strains after digestion with *Sau*3A enzyme. M: molecular size marker, 100-bp ladder (Promega); lane 1, *E. amylovora* PD 2913 (pattern A); lane 2, IVIA 1951-2 (pattern B); lane 3 NCPPB 683 (pattern C); lane 4, PD 4071 (pattern D); lane 5, PD 3368 (pattern E); lane 6, NCPPB 2293 (pattern F); lane 7, NCPPB 2292 (pattern G); lane 8, PD 2915 (pattern H).

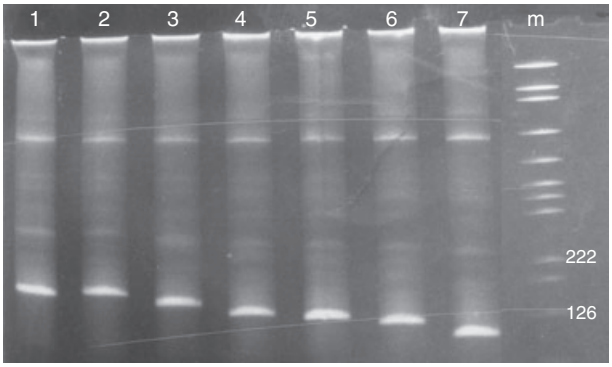

Figure 6 Acrylamide gel showing the detection of single-sequence DNA repeat units in *Erwinia amylovora* strains after the PCR amplification using primers RS1 and RS2c; m, molecular base pairs size marker pGEM (Promega); lane 1, *E. amylovora* H 905; lane 2, PD 3368; lane 3, ICMP 13415; lane 4, ICMP 13417; lane 5, H 902; lane 6, ICMP 13414; lane 7, PD 3891. The band lighter than 222 bp shows the variability in the number of the SSR units.

restriction pattern between the two subgroups. In the present work, the three strains from *Rubus* spp. assessed clustered differently upon UPGMA analysis. It is worth noting that by using REP primer any difference between NCPPB 2292 and NCPPB 2293 was observed. Conversely, the utilization of ERIC primer allowed to discriminate between the two strains. Similar results were obtained by McManus and Jones (1995a) using other *Rubus* strains. Our results are in line also with Kim *et al.* (1995) and Momol *et al.* (1997), founding two *Rubus* subgroups by using the BIOLOG system, and RAPD fingerprinting respectively. How many lineages exist in *E. amylovora* strains isolated from *Rubus* spp. would deserve further studies.

The RFLP analysis of the *Pst*I fragment of the pEA29 plasmid pointed out a higher variability in its length than that previously observed by Lecomte *et al.* (1997). These authors found out three groups of *E. amylovora* strains according to the length of the larger of the fragments observed after the digestion with *Msp*I and *Sau*3A enzymes. The present study showed that the length of the larger fragment spans from around 160 bp to nearly 400 bp. An example of such a relevant variability is given by two strains from *Rubus* spp., each one showing a distinct pattern. Similar results were already obtained by Schnabel and Jones (1998) by observing that the expected band of 307 bp varied in length among the different strains tested. Thus, a clear-cut grouping of *E. amylovora* strains using this technique would not seem reliable.

The relevant variability found in the length of the plasmid fragment was explained by a variation in the number of copies of a SSRs of 8 bp, GAATTACA. Two to 15 SSR units have been described in *E. amylovora* so far (Kim and Geider 1999; Jock *et al.* 2003; Ruppitsch *et al.* 2004). Strain ISF-WC31 from the USA was the fourth strain found so far with two SSR units only. All other strains with that specific SSR number were from

UK (Jock *et al.* 2003). No other isolates were found in Europe with this specific number of SSR units. Traceability of strains through SSR numbers is limited to distinct SSR numbers, (very low or very high SSRs) (Ruppitsch *et al.* 2004). However, our results support the assumption that most Italian *E. amylovora* strains isolated in the Po valley originated from infected plant material from Belgium (Zhang *et al.* 1998; Jock *et al.* 2002). In fact, nearly all Italian strains used in this study had SSR numbers of four to six SSR units similar to strains from Belgium (Jock *et al.* 2003). Therefore, strain UniBaBA6 with eight SSR units and isolated from Apulia region in southern Italy, might have another origin. Strains with that SSR number have been previously found in Germany, England, the Netherlands, Austria, Hungary, the USA and Italy (Kim and Geider 1999; Jock *et al.* 2003; Ruppitsch *et al.* 2004). The same number of SSR units was found in this study for the Hungarian strains were formerly determined by Kim and Geider (1999). This shows that the SSR number is a suitable typing method for strains at least under laboratory conditions. No other correlation due to the SSR number of the investigated strains was observed.

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