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Differentiation of *Erwinia amylovora* strains from Bulgaria by PCR-RFLP analysis

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Abstract Fifty strains of *Erwinia amylovora* isolated in Bulgaria from different host plants and locations as well as in different years were analysed by RFLP analysis of the pEA29 *Pst*I amplified fragment with *Hpa*II. All the strains formed three well-resolved fragments (large—from 365 to 440 bp, medium—about 341 bp and small—about 180 bp).The strains were classified into three RFLP groups based on the polymorphism in the length of the largest fragment. This fragment was of intermediate size for 63% of the strains, and it was the longest (from 410 to 440 bp) for 29% of the strains. The variable region was sequenced for five strains. The DNA sequence analysis confirmed the different size of the largest fragment. Ten or more than ten SSRs were found for the strains in the group with the largest size of the largest fragment. Some correlation between the RFLP profiles and the origin of the strains was revealed. The RFLP profiles displayed stability in certain strains isolated from the same trees and orchards, but in different years. The number of SSRs was different in strains isolated from one and the same host plant, orchard and year, and also in strains isolated from the same host plant and orchard, but in different years. This could indicate that under natural conditions the fire blight symptoms might be caused by a mixture of *E. amylovora* strains with different SSR numbers, and so coexistence of distinguishable strains or a change in the population could be assumed.

Keywords Genetic diversity · *Hpa*II · RFPL of *Pst*I fragment · Short-sequence DNA repeats

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Fire blight, caused by the bacterium *Erwinia amylovora*, is a devastating disease of rosaceous plants (Van der Zwet 1979). Although 129 species of 37 genera of the Rosaceae family are susceptible to the disease worldwide, the most susceptible species are classified in the subfamily Maloideae (Hasler et al. 1996; Nemeth 1999). The disease is of major economic importance in all pear- and apple-growing regions. In Bulgaria, fire blight was first detected in 1990 on quince (Bobev 1990). Since then, the pathogen was progressively observed in different pear and apple-growing areas of the country. The pathogen was recently isolated from new host plants—chokeberry and strawberry (Atanasova et al. 2005). The origin of the initial source of inoculum for fire blight in Bulgaria is not clear.

Assessing the genetic variability of pathogens is important in epidemiology and it is a fundamental prerequisite to develop effective diagnostic protocols (Barionovi et al. 2006). Naturally occurring *E. amylovora* strains carry a low copy number plasmid of 29 kb (pEA29) that can be detected by PCR amplification of the *Pst*I fragment of about 900 bp (Bereswill et al. 1992). Variations in the fragment size were observed in different *E. amylovora* strains, and they were used to classify the strains from central Europe into three types (Lecomte et al. 1997). The cause of the observed size variation is a short sequence DNA repeat (SSR) of eight nucleotides, present in three to 15 copies in the *Pst*I fragment. The number of SSR units has been used for typing of *E. amylovora* strains (Kim and Geider 1999; Jock et al. 2003; Ruppitsch et al. 2004; Barionovi et al. 2006).

After fire blight was detected in Bulgaria, a collection of *E. amylovora* strains was established in our laboratory. It includes strains isolated from nine host plants and from different locations in the country. Several articles have characterised *E. amylovora* strains from some European countries either phenotypically, or genotypically, but only a few papers have described the distribution and initial characterisation of the pathogen in Bulgaria (Garbeva et al. 1996; Bobev et al. 1999; Atanasova et al. 2005). Except for the study of Atanasova et al. (2007), there are no papers on the diversity of Bulgarian strains of *E. amylovora*.

The objective of this study was to differentiate 52 strains of *E. amylovora* (50 of which are Bulgarian isolates, Table 1), previously identified and phenotypically characterised by Atanasova et al. (2005, 2007), by RFLP analysis of the *Pst*I fragment of the plasmid pEA29.

DNA was isolated and purified using a solid-phase DNA isolation kit (STS Ltd., Sofia, Bulgaria), according to the manufacturer's instructions. PCR amplification of a specific 0.9–1.1 kb *Pst*I fragment of plasmid pEA29 for all strains was carried out with the primer pairs pEA29 A and pEA29 B (Bereswill et al. 1992) using the procedure previously described by Atanasova et al. (2005). RFLP analysis of the amplified fragment obtained by the primers mentioned above was carried out to investigate the genetic differentiation among the Bulgarian *E. amylovora* strains. The 900–1,100 bp amplified fragments were digested with the enzyme *Hpa*II (Fermentas, Latvia), chosen on the basis of the restriction maps of the plasmid sequence. The products were analysed in 2.5% agarose gel in TBE buffer. At least three independent restrictions of DNA for each strain were performed.

After the digestion with *Hpa*II (Fig. 1) followed by the analysis of the restriction profiles with the programme Gel Compar, we found three groups of strains (denoted as A, B and C), formed at a similarity level of 86%. The analysis of the restriction profiles of the strains after digestion showed that all the strains contained three well detectable fragments: large (L)—from 365 to 440 bp, medium (M)—about 341 bp, and small (S)—about 180 bp. The fragments M and S were the same size for all strains. The polymorphism among the strains was displayed in the length of the L fragment, and on that basis, the strains were differentiated into the three groups mentioned above. Similar groupings of *E. amylovora* strains was demonstrated earlier by several authors (Lecomte et al. 1997; Schnabel and Jones 1998; Barionovi et al. 2006).

Our group A included 29% of the strains analysed, which exhibited the largest size of the L fragment (between 410 and 440 bp). Strains with similar characteristics were isolated in restricted areas in Europe—in Austria and Germany (Lecomte et al. 1997), and in Hungary and The Netherlands (Barionovi et al. 2006). No data were published on strains with the characteristics of group A isolated from the Balkans. Ten strains out of 15 included in group A were isolated in the period of the first serious disease outbreak in Bulgaria (1995–1997).

Thirty-three of the strains in our collection (63%) belonged to group B, characterised by the medium size of the L fragment (about 400 bp). Strains isolated from the Balkans (Albania, Greece, Turkey and one Bulgarian strain) were included in the analogous RFLP group by Lecomte et al. (1997) and Kim and Geider (1999). Group B was dominant in the Bulgarian populations of the pathogen.

Group C contained five strains (including the type strain *E. amylovora* ATCC 15580) exhibiting the shortest L fragment (below 400 bp). This study confirmed the occurrence in Bulgaria of strains with such a length of the L fragment, which has already been established for two Bulgarian strains by Lecomte et al. (1997).

A correlation was observed between the RFLP profile and the strain origin. It can be seen in Table 1 that all the strains originating from pear, isolated in

Table 1 Sources and year of isolation of *E. amylovora* strains

Strain designation	Host plant	Region of Bulgaria	Year of isolation	RFLP-group	SSRs
*Ea*13	*Pyrus comminis* cv. Cure	Plovdiv orchard	1990	C	
*Ea*29	*Cydonia oblonga* cv. Hemus	Plovdiv orchard	2004	C	
*Ea*1	*Pyrus communis* cv. Butira precoce	Bagrentsi orchard	1995	A	11
*Ea*2	*Pyrus communis* cv. Butira precoce	Bagrentsi orchard	1995	A	
*Ea*3	*Pyrus communis* cv. Butira precoce	Bagrentsi orchard	1995	A	
*Ea*4	*Pyrus communis* cv. Kyustendilska maslovka	Bagrentsi orchard	1995	A	
*Ea*5	*Pyrus communis* cv. Kyustendilska maslovka	Bagrentsi orchard	1995	A	
*Ea*6	*Pyrus communis* cv. Kyustendilska maslovka	Bagrentsi orchard	1995	A	
*Ea*7	*Pyrus communis* cv. Kyustendilska maslovka	Bagrentsi orchard	1995	A	13
*Ea*39	*Malus domestica* cv. Smoothee	Bagrentsi orchard	2000	B	
*Ea*51	*Malus domestica* cv. Smoothee	Bagrentsi orchard	2000	B	
*Ea*40	*Malus domestica* cv. Smoothee	Bagrentsi orchard	2001	B	
Ea42	*Malus domestica* cv. Smoothee	Bagrentsi orchard	2002	B	
*Ea*44	*Malus domestica* cv. Smoothee	Bagrentsi orchard	2003	B	
*Ea*8	*Pyrus comminis* cv. Cure	Jabokrat orchard	1995	A	
*Ea*9	*Pyrus comminis* cv. Cure	Jabokrat orchard	1995	A	
*Ea*10	*Pyrus comminis* cv. Cure	Jabokrat orchard	1995	B	
*Ea*49	*Malus domestica* cv. Smoothee	Jabokrat orchard	2000	B	
*Ea*11	*Malus domestica* cv. Jonathan	Kustendil orchard	1995	A	10
*Ea*16	*Pyrus comminis*	Kustendil orchard	1997	B	
*Ea*17	*Pyrus comminis*	Kustendil orchard	1997	B	
Ea18	*Pyrus comminis*	Kustendil orchard	1997	B	
*Ea*19	*Pyrus comminis* cv. Yubileen dar	Kustendil orchard	1997	B	
*Ea*20	*Pyrus comminis* cv. Yubileen dar	Kustendil orchard	1997	B	
*Ea*21	*Pyrus comminis* cv. Yubileen dar	Kustendil orchard	1997	B	
*Ea*52	*Malus domestica* cv. Melrose	Kustendil orchard	2002	B	
*Ea*54	*Malus domestica* cv. Melrose	Kustendil orchard	2003	B	
*Ea*55	*Malus domestica* cv. Melrose	Kustendil orchard	2004	B	8
*Ea*32	*Pyrus comminis* cv. Cure	Kostinbrod orchard	1999	A	
*Ea*33	*Pyrus comminis* cv. Beurre Giffard	Kostinbrod orchard	1999	A	
*Ea*237	*Fragaria moshata*	Slaviantsi orchard	1999	B	
*Ea*238	*Fragaria moshata*	Slaviantsi orchard	1999	B	
*Ea*236	*Malus domestica* cv. Jonathan	Slaviantsi orchard	2001	A	
Ea30	*Pyrus comminis* cv. Beurre Hardy	Dupnitsa orchard	1999	A	12
*Ea*34	*Pyrus comminis* cv. Beurre Hardy	Dupnitsa orchard	2002	A	
*Ea*36	*Crataegus* spp.	Vitosha mountain	1999	B	
*Ea*246	*Pyrus* spp.	Petrich	2002	B	
*Ea*22	*Cydonia oblonga* cv. Asenitsa	Sofia region	2002	B	
*Ea*23	*Cydonia oblonga* cv. Asenitsa	Sofia region	2003	B	
*Ea*24	*Cydonia oblonga* cv. Asenitsa	Sofia region	2004	B	
*Ea*25	*Pyracantha coccinea*	Sofia	2003	B	
*Ea*26	*Pyracantha coccinea*	Sofia	2004	B	
*Ea*27	*Pyracantha coccinea*	Sofia	2004	B	
Ea28	*Cotoneaster integerrimus*	Sofia	2004	C	

Table 1 (continued)

Strain designation	Host plant	Region of Bulgaria	Year of isolation	RFLP-group	SSRs
*Ea*244	*Fragaria ananassa*	Lovech orchard	2002	B	
*Ea*245	*Fragaria ananassa*	Suhindol orchard	2003	B	
*Ea*15	*Aronia melanocarpa*	Plovdiv region	2004	B	
*Ea*14	*Aronia melanocarpa*	Central Old mountain	2004	B	
*Ea*31	*Cydonia oblonga* cv. Albena	Dolna Banya orchard	2004	B	
*Ea*1C	*Pyrus communis*	Serbia	Unknown	C	
*Ea*2C	*Pyrus communis*	Serbia	Unknown	B	
*Ea*ATCC15580	*Pyrus communis*	–		C	

1995 in Bagrentsi, belong to group A, while the strains from the same orchard, but isolated from apple in the period 2000–2003, belong to group B. This might cause speculation about two possible sources of the initial inoculum. A similar speculation could also concern the strains from Jabokrat. All the strains from Kustendil except the one isolated in 1995 formed a cluster in group B, regardless of their host and the year of isolation. Two out of the three strains in group C were isolated from Plovdiv. The small number of strains isolated from the remaining sources did not allow clear conclusions to be made. However, some data from the analyses suggest that the strains from several of the orchards examined could be of common origin.

Some temporal stability of the RFLP profiles was established. It can be seen that strains *Ea*40, *Ea*42 and *Ea*44 possess the same RFLP profile as strains *Ea*39 and *Ea*51, isolated from the same tree and orchard but earlier in time. The situation was identical with strains *Ea*52, *Ea*54 and *Ea*55; *Ea*30 and *Ea*34; *Ea*25, *Ea*26 and *Ea*27. Similar stability was also observed in the laboratory. In routine laboratory procedures, the strains were transferred many times from solid into liquid media, stored at −20°C in glycerol, and then DNA was isolated, amplified, and subjected to restriction with *Hpa*II. In all cases the RFLP patterns of the strains remained stable.

Different authors explained the variability in the length of the RFLP fragments by a variation in the number of SSR units. Variability in SSR size has been shown by Kim and Geider (1999), Jock et al. (2003) and Ruppitsch et al. (2004). All Italian strains tested by Barionovi et al. (2006) had SSR numbers of four

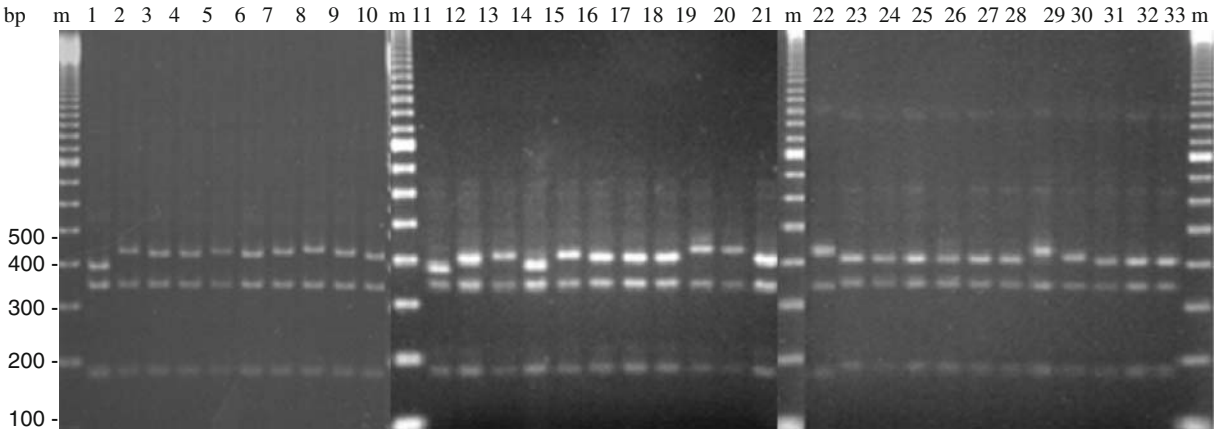

Fig. 1 Representative RFLP patterns of Bulgarian *Erwinia amylovora* strains obtained by restriction analysis of the pEA29 *Pst*I amplified fragments with *Hpa*II. *m* DNA marker 100 bp (Amersham, Biosciences); *1*, type culture of *E. amylovora* ATCC 15580; *2*, *Ea*1; *3*, *Ea*2; *4*, *Ea*3; *5*, *Ea*4; *6*, *Ea*5; *7*, *Ea*6; *8*, *Ea*7; *9*, *Ea*8; *10*, *Ea*9; *11*, *Ea*1C; *12*, *Ea*2C; *13*, *Ea*10; *14*, *Ea*13; *15*, *Ea*16; *16*, *Ea*18; *17*, *Ea*19; *18*, *Ea*21; *19*, *Ea*30; *20*, *Ea*33; *21*, *Ea*246; *22*, *Ea*11; *23*, *Ea*36; *24*, *Ea*42; *25*, *Ea*44; *26*, *Ea*51; *27*, *Ea*54; *28*, *Ea*55; *29*, *Ea*236; *30*, *Ea*237; *31*, *Ea*244; *32*, *Ea*245; *33*, *Ea*247

to six, similar to strains from Belgium (Jock et al. 2003). In the light of this information, all *Pst*I fragments from five strains were cloned into vector pMOS*Blue* (Amersham, Biosciences, Vienna, Austria) and sequenced with the ABI 3130 sequencer (MWG-Biotech). The sequences of these five strains were compared by means of PC/Gene programme Clustal W Multiple Sequence Alignments Version 1.82 (Thompson et al. 1994) with the corresponding sequences of *E. amylovora* 88 obtained from GenBank (Accession Number AF 264948). Four of the sequenced strains (*Ea*1, *Ea*7, *Ea*11, *Ea*30) belonged to RFLP group A (with the largest size of the L fragment), and strain *Ea*55 was from group B (with medium size of the L fragment). The numbers of SSRs in the sequenced strains are presented in Table 1. Eight SSRs were found in strain *Ea*55 (Accession number EU725790). Ten to 13 SSRs were observed for the sequenced strains from the group with the largest size of the L fragment (*Ea*11—accession number EU725787; *Ea*1—accession number EU725789; *Ea*30—accession number EU725791, and *Ea*7—accession number EU725788).

Besides the different SSR numbers, there were certain dissimilarities among these five strains, as well as between these and the reference strain, in the bases out of the variable region. Generally, strains *Ea*55, *Ea*11 and *Ea*30, which differed with each other only by their repeats numbers, formed a group unlike the other two strains and the strain from GenBank. Obviously, these differences should become the object of additional studies.

Different numbers of SSRs were found for strains *Ea*1 and *Ea*7, isolated from one and the same host plant, orchard and in the same year, and for strains *Ea*11 and *Ea*55 isolated from the same host plant and orchard, but in different years. This could indicate that under natural conditions the fire blight symptoms might be caused by a mixture of *E. amylovora* strains with different SSR numbers, and so coexistence of distinguishable strains or a change in the population of the pathogen could be assumed.

Many authors have analysed the stability of SSRs, and in some cases it was proven, but in other cases it was not observed. As mentioned previously, our strains preserved their RFLP patterns after a number of laboratory manipulations, which is indirect evidence for SSR stability. A number of strains isolated from the same trees and orchards, but several years later than the sequenced strains, were subjected to PCR with primers RS1 and RS2c and NovaTaq PCR kit (Merck) to access their SSR numbers (data not shown). Some isolates were found with the same SSR numbers as in the sequenced strains, but some strains had different SSR numbers. We could not, however, conclude on SSR stability because differences of one or two repeats could not be readily detected without sequencing.

This work presents, for the first time, Bulgarian strains with eight, ten, 12 and 13 SSR units, and confirms the existence of strains with 11 SSRs previously found by Kim and Geider (1999). The data obtained in our investigation expands the knowledge on the overall genetic diversity of *E. amylovora* using the characteristics of Bulgarian strains. This study is the first characterisation of the genetic diversity of Bulgarian populations of *E. amylovora*. Comparing our results with those of other authors, at least three sources of inoculum for the Bulgarian population of *E. amylovora* could be suggested.

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