Characterization of *Erwinia amylovora* Strains from Bulgaria by Pulsed-Field Gel Electrophoresis

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The aim of this study was to characterize genetically Bulgarian *Erwinia amylovora* strains using pulsed-field gel electrophoresis (PFGE) analysis. Fifty *E. amylovora* strains isolated from different hosts, locations, as well as in different years were analysed by PFGE after *Xba*I, *Spe*I, and *Xho*I digestion of the genomic DNA. The strains were distributed into four groups according to their *Xba*I-generated profile. About 82% of the strains displayed a PFGE profile identical to that of type Pt2. Three strains belonged to the Central Europe Pt1 type. Two new PFGE profiles, not reported so far, were established – one for a strain isolated from *Malus domestica* and another for all *Fragaria* spp. strains. The same grouping of the strains was obtained after analysis of the *Spe*I digestion patterns. On the basis of PFGE profiles, after *Xba*I and *Spe*I digestion, a genetic differentiation between the strains associated with subfamily Maloideae and subfamily Rosoideae was revealed. The presence of more than one PFGE profile in the population of *E. amylovora* in Bulgaria suggests a multiple source of inoculum.

Key words: Differentiation, *Erwinia amylovora*, PFGE

Introduction

Fire blight, a disease that is responsible for serious fruit losses of rosaceous plants, is caused by the Gram-negative bacterium *Erwinia amylovora*. The disease is indigenous to North America but has spread to many countries in the world (Van der Zwet, 1996). In Bulgaria, fire blight was first detected in 1990 on quince (Bobev, 1990). Since then, the pathogen has been progressively observed in different regions of the country mainly on pear, apple, and quince trees, strawberry, chokeberry, cotoneaster, and pyracantha (Atanasova *et al*., 2005, 2007). The transmission and spread of *E. amylovora* in the country is the result of the import of contagious plants from Western Europe, as well as from the natural dissemination of the bacterium and the absence of an effective control of the disease. The data from phenotypic assays suggested that *E. amylovora* is a homogeneous species. The recent historical distribution of the pathogen may also be a reason for this homogeneity in the genomes of individual strains (Zhang and Geider, 1997). However, several molecular techniques such as polymerase chain reaction (PCR)-ribotyping (McManus and Jones, 1995; Jeng *et al*., 1999), random amplified polymorphic DNA (RAPD) (Momol *et al*., 1995), restriction fragment length polymorphism (RFLP) (Lecomte *et al*., 1997; Kim and Geider, 1999; Jock *et al*., 2003; Ruppitsch *et al*., 2004; Barionovi *et al*., 2006; Atanasova *et al*., 2009), amplified fragment length polymorphism (AFLP) (Rico *et al*., 2004; Donat *et al*., 2007), and pulsed-field gel electrophoresis (PFGE) (Zhang and Geider, 1997; Zhang *et al*., 1998; Jock *et al*., 2002a; Jock and Geider, 2004; Halupecki *et al*., 2006; Donat *et al*., 2007) have proven useful in the determination of intraspecific diversity within strains from different geographical origins and hosts. Some of these approaches like RAPD and

Abbreviations: AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA.

PCR-ribotyping allowed differentiation between Maloideae and Rosoideae strains (Momol *et al.*, 1995; McManus and Jones, 1995; McGhee *et al.*, 2002).

Macrorestriction analysis of the bacterial genome revealed several closely related but distinguishable pattern types for *E. amylovora*, which were used to follow up the spread of the disease through Europe, the Mediterranean region, and the Balkans (Jock *et al.*, 2002a). The PFGE profiles obtained with *Xba*I- digested chromosomal DNA of *E. amylovora* were used to group strains from different geographic regions (Zhang and Geider, 1997; Zhang *et al.*, 1998; Jock *et al.*, 2002b). On the basis of this analysis, six PFGE types were established – Pt1, Pt2, Pt3, Pt4, Pt5, and Pt6 (Jock *et al.*, 2002a). The patterns differ in the shift or the lack of one band. Normally, one typical PFGE pattern is found when fire blight is established in a certain country. Only two strains isolated from Bulgaria have been subjected to macrorestriction analysis and were established to have Pt2 and Pt5 PFGE patterns (Zhang *et al.*, 1998; Jock *et al.*, 2002a). The genetic diversity of the *E. amylovora* population in Bulgaria has not been studied in detail, and very little is known about the genetic variability of Bulgarian strains.

The aim of this work was to study the genetic diversity among fifty strains of *E. amylovora*, isolated from different plants and locations in Bulgaria as well as in different years, by PFGE analysis.

Material and Methods

Bacterial strains

Fifty strains previously identified as *E. amylovora* (Bogatzevska, 2000; Atanasova *et al.*, 2005, 2007; Kabadjova-Hristova *et al.*, 2006) were used in this study. The type strain *E. amylovora* ATCC 15580 and strain 2C (Serbian isolate which was kindly provided to us by Prof. I. Kiryakov, Dobroudja Agricultural Institute, General Toshevo, Bulgaria) were used as controls. The origin and year of isolation of the strains are listed in Table I.

PFGE analysis

The PFGE analysis of chromosomal DNA of *E. amylovora* was performed as described by Zhang and Geider (1997) with some modifications. Briefly, the cultures were cultivated in LB broth at 27 °C for about 24 h up to an optical density of 0.6 ($A_{600\,nm}$). Cells from 2 ml broth were harvested by centrifugation and washed twice in SEP buffer (25 mM sodium phosphate buffer, pH 8.0, with 0.3 M sucrose, 25 mM EDTA). The cells were resuspended in 1 ml SEP buffer and mixed at 45 °C with an equal volume of 1.5% low-melting point agarose (Sigma-Aldrich Chemie, Steinheim, Germany) prepared in SEP buffer. The agarose-cell suspension was poured into disposable Bio-Rad (Hercules, CA, USA) plug molds (10/5/1.5 mm) and allowed to solidify. Lysis of the agarose-embedded cells was performed in lysis buffer (1 mg ml^{-1} proteinase K, 1 mM $CaCl_2$, 1% lauroylsarcosine, 0.25 M EDTA, 10 mM glycine, pH 9.5) for 48 h followed by two successive washes in 10 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. A quarter of a plug was digested overnight with either of the restriction enzymes *Xba*I, *Spe*I, or *Xho*I (20 U). After digestion, the resulting DNA fragments were resolved by PFGE analysis with a Bio-Rad CHEF-DR II apparatus for 24 h at 14 °C with ramping at 5 V cm^{-1} in 1% agarose gel with HEPES buffer and a ramping time of 5–30 s. All strains studied were assayed, at least twice, from different DNA extractions. The positions of the bands after digestion were compared with GelCompar (Applied Maths, Koptrijk, Belgium) software.

Results and Discussion

To estimate the possible diversity of *E. amylovora* in Bulgaria, we used strains isolated from different host plants, orchards, and years (Table I). The samples were derived from blight-infected orchards with pear, apple, and quince trees, as well as from strawberry plantations, aronia, hawthorn, and ornamental plants. Restriction digests with three enzymes (*Xba*I, *Spe*I, and *Xho*I) and subsequent PFGE analyses of genomic DNA were applied in this study to fifty-two *E. amylovora* strains. Fifty of them were Bulgarian isolates.

PFGE analysis of XbaI-digested chromosomal DNA

The restriction profiles after *Xba*I digestion (Fig. 1) were compared in order to find diversity that can be used for differentiation among the strains. The molecular mass of each band was determined. Four distinct patterns were distinguished

Table I. Sources, year of isolation, and PFGE patterns of *E. amylovora* strains.

Strain designation	Host plant	Location	Year	*Xba*I PFGE group	*Spe*I PFGE group
*Ea*1, *Ea*2, *Ea*3, *Ea*4, *Ea*5, *Ea*6, *Ea*7	*Pyrus communis*	42°16'44.73"N 22°45'36.33"E	1995	II (Pt2)	II
*Ea*39	*Malus domestica*	"	2000	IV (new Pt type)	IV
*Ea*51	*Malus domestica*		2000	II (Pt2)	II
*Ea*40	*Malus domestica*	"	2001	II (Pt2)	II
*Ea*42	*Malus domestica*	"	2002	II (Pt2)	II
*Ea*44	*Malus domestica*	"	2003	II (Pt2)	II
*Ea*8, *Ea*9, *Ea*10	*Pyrus communis*	42°18'06.07"N 22°45'24.73"E	1995	II (Pt2)	II
*Ea*49	*Malus domestica*	"	2000	II (Pt2)	II
*Ea*11	*Malus domestica*	42°16'52.47"N 22°41'17.66"E	1995	II (Pt2)	II
*Ea*16, *Ea*17, *Ea*18, *Ea*19, *Ea*20, *Ea*21	*Pyrus communis*	"	1997	II (Pt2)	II
*Ea*52	*Malus domestica*	"	2002	II (Pt2)	II
*Ea*54	*Malus domestica*	"	2003	II (Pt2)	II
*Ea*55	*Malus domestica*	"	2004	II (Pt2)	II
*Ea*13	*Pyrus communis*	42°08'37.83"N 24°44'58.42"E	1990	I (Pt1)	I
*Ea*15	*Aronia melanocarpa*	"	2004	I (Pt1)	I
*Ea*29	*Cydonia oblonga*	"	2004	I (Pt1)	I
*Ea*14	*Aronia melanocarpa*	42°55'46.99"N 25°52'38.92"E	2004	II (Pt2)	II
*Ea*31	*Cydonia oblonga*	42°18'40.81"N 23°45'56.49"E	2004	II (Pt2)	II
*Ea*22	*Cydonia oblonga*	42°39'17.58"N 23°16'42.14"E	2002	II (Pt2)	II
*Ea*23	*Cydonia oblonga*	"	2003	II (Pt2)	II
*Ea*24	*Cydonia oblonga*	"	2004	II (Pt2)	II
*Ea*25, *Ea*26, *Ea*27	*Pyracantha coccinea*	42°41'47.37"N 23°19'33.64"E	2003	II (Pt2)	II
*Ea*28	*Cotoneaster integerrimus*	"	2004	II (Pt2)	II
*Ea*30	*Pyrus communis*	42°15'44.87"N 23°06'31.51"E	1999	II (Pt2)	II
*Ea*34	*Pyrus communis*	"	2002	II (Pt2)	II
*Ea*32, *Ea*33	*Pyrus communis*	42°49'06.29"N 23°13'29.91"E	1999	II (Pt2)	II
*Ea*247	*Fragaria moshata*	"	1999	III (new Pt type)	III
*Ea*36	*Crataegus* sp.	42°32'01.28"N 23°21'58.12"E	1999	II (Pt2)	II
*Ea*236	*Malus domestica*	42°45'52.67"N 26°43'42.46"E	2001	II (Pt2)	II
*Ea*237, *Ea*238	*Fragaria moshata*	"	1999	III (new Pt type)	III
*Ea*244	*Fragaria ananassa*	43°08'05.42"N 24°43'02.14"E	2002	III (new Pt type)	III
*Ea*245	*Fragaria ananassa*	43°11'14.96"N 25°10'22.44"E	2003	III (new Pt type)	III
*Ea*246	*Pyrus* sp.	41°23'53.24"N 23°12'24.88"E	2002	II (Pt2)	II
*Ea*ATCC15580	*Pyrus communis*			I (Pt1)	I
*Ea*2C	*Pyrus communis*		Unknown	II (Pt2)	II

after *Xba*I digestion and PFGE (Table I). Single strains, including the type culture of *E. amylovora*, isolated from three different host plants, formed the first group, displaying the PFGE pattern Pt1. It is interesting to note that all strains had been isolated from the Plovdiv region (Atanasova *et*

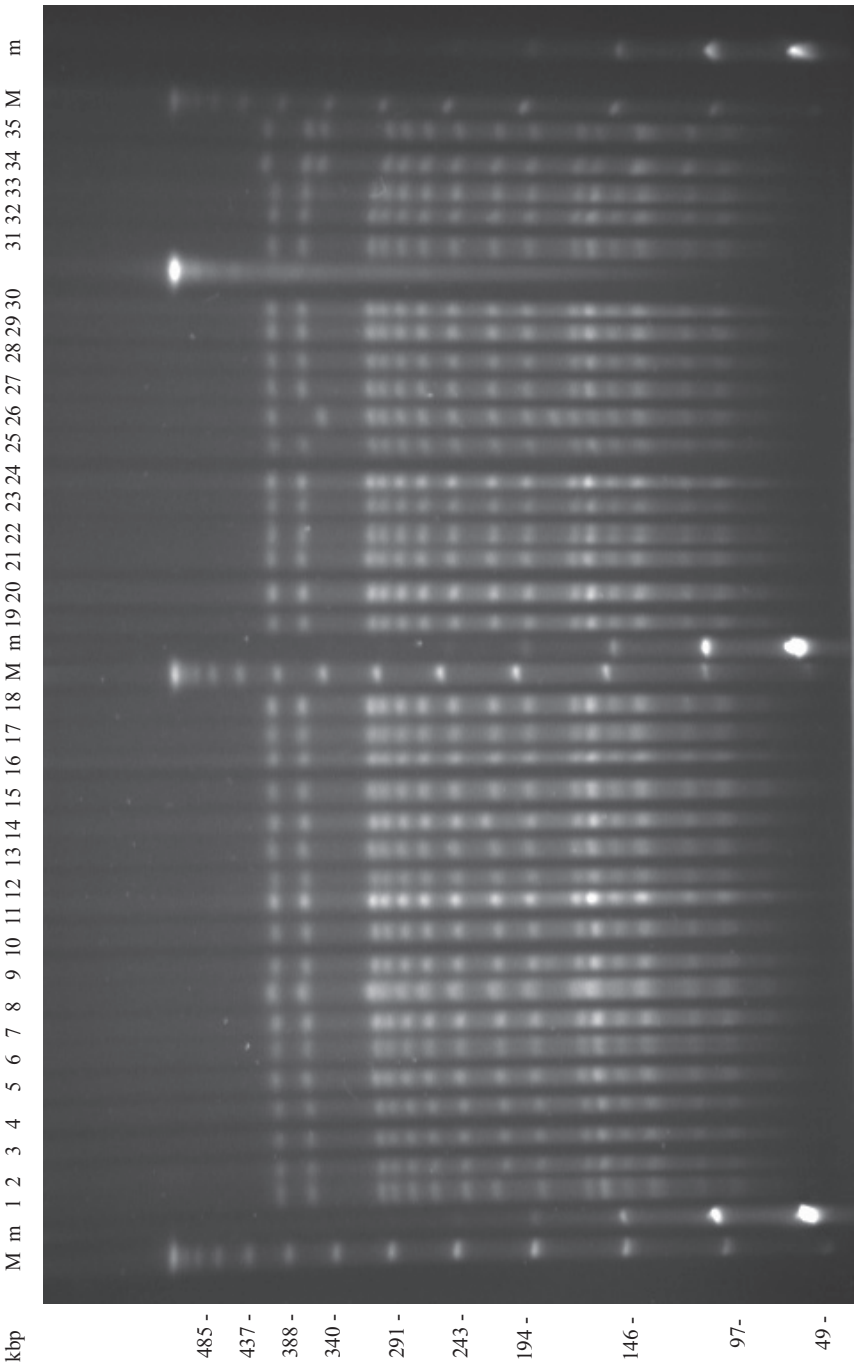

Fig. 1. Representative PFGE patterns of Bulgarian *Erwinia amylovora* strains after restriction with *Xba*I. Lane M, pulse marker, 50–1000 kb (Sigma); lane m, DNA marker (γ-phage DNA, 0.1–200 kb, Sigma); lane 1, type culture of *E. amylovora* ATCC 15580; lane 2, *Ea*1; lane 3, *Ea*2; lane 4, *Ea*3; lane 5, *Ea*4; lane 6, *Ea*5; lane 7, *Ea*6; lane 8, *Ea*7; lane 9, *Ea*8; lane 10, *Ea*9; lane 11, *Ea*10; lane 12, *Ea*11; lane 13, *Ea*12; lane 14, *Ea*13; lane 15, *Ea*16; lane 16, *Ea*17; lane 17, *Ea*18; lane 18, *Ea*19; lane 19, *Ea*20; lane 20, *Ea*21; lane 21, *Ea*30; lane 22, *Ea*32; lane 23, *Ea*33; lane 24, *Ea*34; lane 25, *Ea*36; lane 26, *Ea*39; lane 27, *Ea*40; lane 28, *Ea*42; lane 29, *Ea*44; lane 30, *Ea*51; lane 31, *Ea*54; lane 32, *Ea*55; lane 33, *Ea*236; lane 34, *Ea*237; lane 35, *Ea*238. The lane with unsuccessful DNA digestion is not numbered.

al., 2009). For Bulgaria and the Balkan region in general this profile has not been reported so far. In the investigations of Zhang and Geider (1997), Zhang *et al*. (1998), Jock *et al*. (2002b), and Donat *et al.* (2007) the PFGE type Pt1 was found for the strains of *E. amylovora* isolated in England, Central Europe, Northern France, and Spain. The presence of this profile in Bulgaria could possibly be explained by plant imports, accidental introduction, or introduction by natural vectors of the pathogen from areas where fire blight was caused by strains displaying type Pt1.

The majority of the Bulgarian strains (82%) as well as the Serbian strain *Ea*2C displayed a PFGE profile identical with the type Pt2, and formed the second group. The strains of this major group originated from different locations, host plants, and orchards and were isolated in different years, which indicates genomic stability, as well as homogeneity of the Bulgarian population of *E. amylovora*. Additionally, the strains of this group possessed different RFLP profiles of the pEA29 plasmid *Pst*I-amplified fragment digested with *Hpa*II and a different number of SSR (short sequence repeats) – 8, 10, 11, 12, and 13 repeats (Atanasova *et al*., 2009). Halupecki *et al*. (2006) found that the type Pt2 was characteristic for the strains of *E. amylovora* isolated in Croatia. Zhang *et al*. (1998) observed that the two Bulgarian strains included in their work possessed profiles previously found for Mediterranean strains, respectively, Pt2 and Pt5.

All five isolates from *Fragaria* spp. (subfamily Rosoideae) had a *Xba*I digest pattern, which differed greatly from that of the other Bulgarian strains, as well as from all profiles reported for *E. amylovora* so far, and were included in the third group. This profile is characterized by the shift of one band from 400 kbp to about 414 kbp, the appearance of one new 348-kbp band, and the lack of two bands (146 kbp and 299 kbp). All these strains showed the same RFLP profile of the pEA29 plasmid *Pst*I-amplified fragment digested with *Hpa*II (Atanasova *et al*., 2009). Zhang and Geider (1997) described a PFGE profile of a strawberry *E. amylovora* strain identical to the Maloideae strains and different from the profile established by us, but emphasized that the strain was isolated from plantations near apple orchards. The *E. amylovora* strains from *Fragaria* spp. used in our work had been isolated from a strawberry plantation totally destroyed by the pathogen. The pathogenicity of the strains was confirmed by vacuum infiltration of young strawberry plants with bacterial suspension which resulted in typical fire blight symptoms (Bogatzevska, 2000; Atanasova, 2006). The occurrence of a new genetic pattern of strawberry isolates of *E. amylovora* reflects the difference between Rosoideae and Maloideae strains.

One strain (*Ea*39) isolated from *Malus domestica* (cv. Smoothee) possessed a profile distinctly different from all other PFGE patterns described so far. The PFGE pattern of this strain is characterized by the shift of one band, characteristic for the main Pt2 profile, from 364 kbp to about 346 kbp, and by the appearance of a new band. This suggested a shift of the site of restriction resulting in the generation of a new 177-kbp fragment instead of 159 kbp as it was in the other profiles (a fusion of fragments at 159 kbp and 18 kbp to a band at 177 kbp). Some authors explained similar observations by a spontaneous mutation affecting a single DNA fragment (Zhang and Geider, 1997; Zhang *et al*., 1998).

We did not find strains with the profile type Pt5, previously reported for one Bulgarian strain by Zhang *et al*. (1998).

The PFGE pattern identified for each strain was reproducible in all cases.

The restriction with *Xba*I followed by PFGE analysis revealed relative genetic homogeneity of the Bulgarian population of *E. amylovora* – 82% of the strains possessed the same profile, which was identical to the known Pt2 profile. Nevertheless, this analysis displayed the genetic diversity in the population, since the profile Pt1 and two new profiles were established. These results reveal the possibility that strains originating from plants of the subfamilies Maloideae and Rosoideae can be distinguished genetically on the basis of their PFGE profiles. The comparison of our results with the data published by other authors allows us to suggest that the main pathogen population was probably introduced in Bulgaria from the Eastern Mediterranean, where type Pt2 is characteristic.

PFGE analysis of SpeI-digested chromosomal DNA

Genomic DNA from the same strains was analysed by PFGE after digestion with *Spe*I (Fig. 2). The comparison of the positions of the resulting bands by GelCompar also distributed the strains

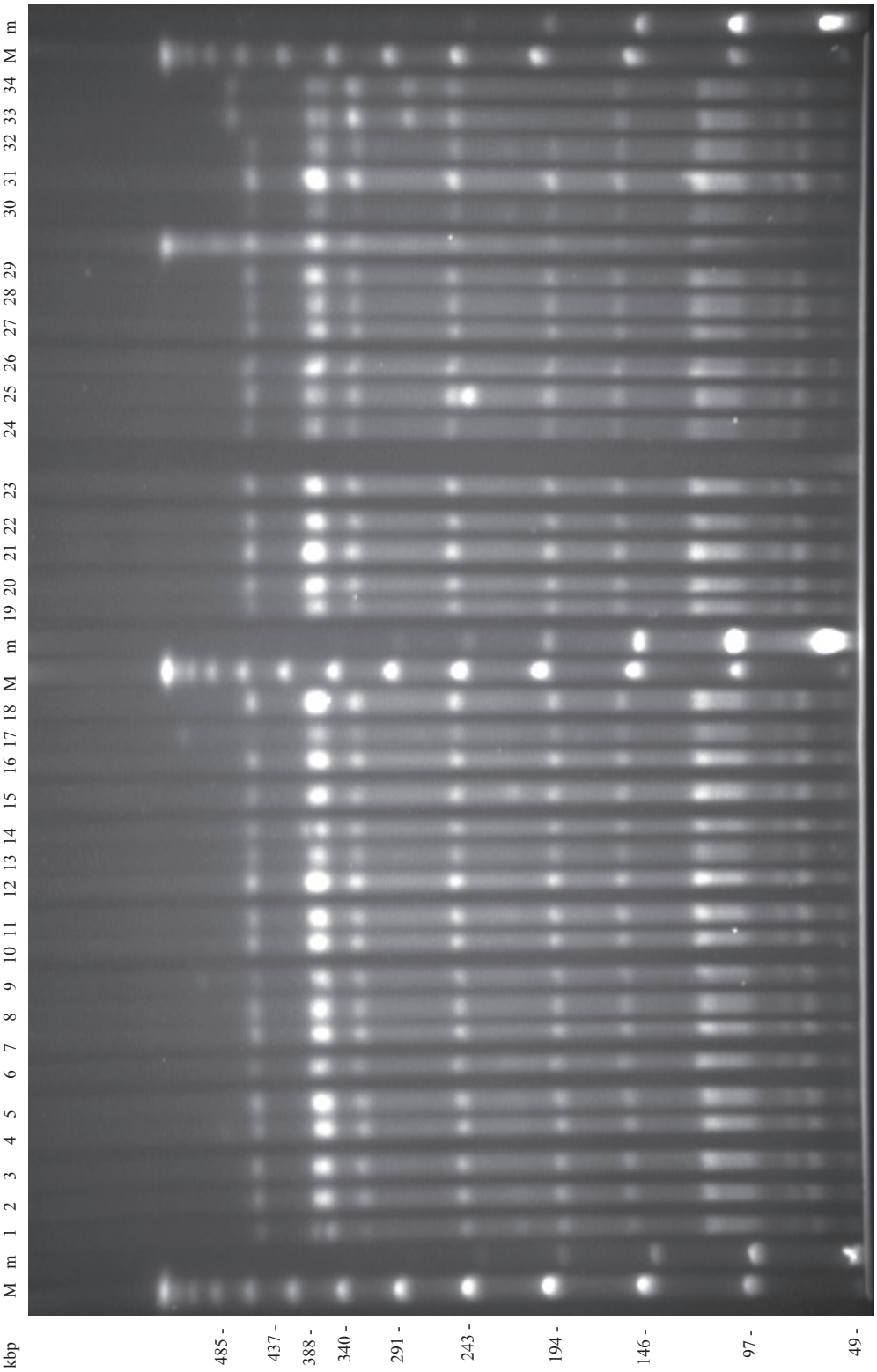
m M 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 m M 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 m M
kbp
485 -
437 -
388 -
340 -
291 -
243 -
194 -
146 -
97 -
49 -

Fig. 2. Representative PFGE patterns of Bulgarian *Erwinia amylovora* strains after restriction with *Spe*I. Lane M, pulse marker, 50–1000 kb (Sigma); lane m, DNA marker (γ-phage DNA, 0.1–200 kb, Sigma); lane 1, type culture of *E. amylovora* ATCC 15580; lane 2, *Ea*1; lane 3, *Ea*2; lane 4, *Ea*3; lane 5, *Ea*4; lane 6, *Ea*5; lane 7, *Ea*6; lane 8, *Ea*7; lane 9, *Ea*8; lane 10, *Ea*9; lane 11, *Ea*10; lane 12, *Ea*11; lane 13, *Ea*12; lane 14, *Ea*13; lane 15, *Ea*16; lane 16, *Ea*17; lane 17, *Ea*18; lane 18, *Ea*19; lane 19, *Ea*20; lane 20, *Ea*21; lane 21, *Ea*30; lane 22, *Ea*32; lane 23, *Ea*33; lane 24, *Ea*36; lane 25, *Ea*39; lane 26, *Ea*40; lane 27, *Ea*42; lane 28, *Ea*44; lane 29, *Ea*51; lane 30, *Ea*54; lane 31, *Ea*55; lane 32, *Ea*236; lane 33, *Ea*237; lane 34, *Ea*238. The lane with unsuccessful DNA digestion is not numbered.

into four groups according to their PFGE profiles (Table I). The first group included three strains as well as the type culture of *E. amylovora*. The second group, which was the major one, included 82% of the strains. In this group a multiple band of about 350 kbp was observed. Strain *Ea*39, as well as the five Rosoideae strains separated from all other isolates into a third and a fourth group, respectively. The strain structure of the *Spe*I groups was identical to that formed with *Xba*I. The *Spe*I PFGE patterns of the strains were characterized by a smaller number of fragments in comparison to those with *Xba*I and overlapping bands corresponding to about 350 kbp. The restriction with *Spe*I also allowed the differentiation between Rosoideae and Maloideae strains.

The third restriction enzyme used in this study, *Xho*I, generated identical macrorestriction profiles for all studied isolates, including the type strain of *E. amylovora* (results not shown).

In conclusion, this work revealed the relative genetic homogeneity of the Bulgarian population of *E. amylovora*, since about 82% of the strains isolated from host plants from the subfamily Maloideae showed identical PFGE patterns. For a few Maloideae strains two additional profiles were established – type Pt1 (after *Xba*I digestion) and, so far, one unknown pattern for *E. amylovora* (*Malus domestica*-derived isolate). All *Fragaria* spp. isolates clustered into a separate group demonstrating the genetic difference between the strains isolated from Maloideae and Rosoideae plants on the base of their *Xba*I and *Spe*I restriction profiles. Our study extends the data on the genetic diversity of *E. amylovora* and, based on the results obtained, a multiple source of inoculum of the Bulgarian population of *E. amylovora* can be assumed.

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