

GENOMIC ANALYSIS BY PULSED-FIELD GEL ELECTROPHORESIS OF *ERWINIA AMYLOVORA* STRAINS FROM THE MEDITERRANEAN REGION INCLUDING ITALY

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SUMMARY

Erwinia amylovora strains from the Mediterranean region including Italy, and from eastern Europe were assayed for their PFGE patterns after an *Xba*I digest of the genomic DNA. The strains from eastern Europe and the Mediterranean region were placed into the same group (Pt2), although a strain from Bulgaria and two strains from Israel carried an RFLP-pattern which distinguished them from the other strains in that regions. Strains from Austria and Czechia belonged to the central European type (Pt1), whereas strains from Italy could be divided into three groups, *i.e.* with patterns observed before for strains from northern France (Pt3), the Mediterranean region (Pt2), and central Europe (Pt1). Grouping of *E. amylovora* strains from northeastern Italy to strains from northern France was confirmed by PFGE analysis after *Spe*I digests. With an increasing number of *E. amylovora* strains isolated in the Mediterranean region, their differentiation by PFGE could possibly give clues about the distribution of fire-blight in the surrounding European countries.

RIASSUNTO

ANALISI GENOMICA MEDIANTE GEL ELETTROFORESI IN CAMPO PULSATO DI CEPPI DI *ERWINIA AMYLOVORA* PROVENIENTI DALLA REGIONE MEDITERRANEA COMPREENDE L'ITALIA. I profili PFGE di ceppi di *Erwinia amylovora*, isolati nella regione mediterranea comprendente l'Italia e l'Europa orientale, sono stati analizzati dopo digestione del DNA genomico con endonucleasi *Xba*I. Un ceppo di origine bulgara e due ceppi provenienti da Israele hanno evidenziato un profilo PFGE distinguibile da quello degli altri ceppi isolati nell'Europa orientale e nella regione mediterranea, collocati invece nello stesso gruppo (tipo Pt2). I ceppi di origine Austriaca e Ceca hanno mostrato un profilo

centro europeo (Pt1), mentre i ceppi isolati in Italia sono stati suddivisi in tre gruppi, con profili analoghi a quelli attribuiti a ceppi isolati nella Francia settentrionale (Pt3), nella regione mediterranea (Pt2) e nell'Europa centrale (Pt1). L'analisi PFGE dei prodotti di digestione con *Spe*I ha confermato l'appartenenza allo stesso gruppo dei ceppi di *E. amylovora* isolati nell'Italia nord-orientale e nella Francia settentrionale. La differenziazione mediante PFGE di un maggior numero di ceppi di *E. amylovora* isolati nella regione mediterranea potrebbe verosimilmente fornire indicazioni sulla distribuzione del colpo di fuoco nei paesi europei limitrofi.

Key words: fire-blight, differentiation of European strains, spread of disease, PFGE.

INTRODUCTION

Fire-blight, a pome-fruit tree disease caused by the Gram-negative bacterium *Erwinia amylovora*, has spread into Europe since it appeared in England in 1957 (Van der Zwet, 1996; Paulin, 1997). In the last 25 years, fire-blight was reported in eastern Europe, from Greece, Armenia, Bulgaria, former Yugoslavia and from Romania. Recently, its presence was confirmed in Spain (de la Cruz Blanco, 1996), Albania (Pace and Mazzucchi, 1996), Hungary (Hevesi, 1996), Croatia (B. Cvietkovik, personal communication) and Czechia (V. Kudela, personal communication). In Italy, it was first reported in summer 1990 in pear orchards of the Apulia region, southern Italy (Cariddi, 1990); during summer 1991, a small disease focus occurred in Sicily (D'Anna *et al.*, 1994). In early fall 1994, fire-blight was found near Bologna (Emilia-Romagna region, north-eastern Italy) (Finelli *et al.*, 1996). Early 1998, the officially detected foci were more than 700 in five districts of the Emilia-Romagna region (Regional Phytosanitary Service, Bologna, personal communication).

For specific detection and identification of the fire-blight pathogen *E. amylovora*, various tools have been developed such as semi-selective media, serological assays, DNA hybridization and PCR techniques. DNA hybridization was performed using DNA fragments from

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the 29-kb plasmid, common to all *E. amylovora* strains (Falkenstein *et al.*, 1988). Fast, specific and sensitive PCR assays were first based on information from plasmid pEA29 (Bereswill *et al.*, 1992) and were recently supplemented with primers from the chromosomal region for amylovoran synthesis (*ams*), the band pattern from 16S rDNA amplification with a subsequent *Hae*III digest, and AP-PCR both with primers from transposon Tn5 (Bereswill *et al.*, 1995), and with primers derived from a random genomic fragment of *E. amylovora* DNA (Guilford *et al.*, 1996). Nested PCR, PCR dot-blot and reverse hybridization with information from plasmid pEA29 made detection of *E. amylovora* even more sensitive (McManus and Jones, 1995a); moreover, mass tests on plant propagation material can be speeded up using automated immunoenzymatic detection of PCR amplicons, captured by oligo-probe hybridization, in ELISA-microplates, without carry-over risks and specificity problems such as detection of primer oligomers (M. Merighi *et al.*, unpublished).

The data from classical assays suggested that *E. amylovora* is a homogeneous species, supported by earlier observations from serological (Vantomme *et al.*, 1982) and biochemical studies (Dye, 1968), DNA hybridization (Brenner *et al.*, 1974) and host range tests (Starr and Chatterjee, 1972).

Recently, several approaches were tried to differentiate strains of the pathogen such as the use of random primers (Momol *et al.*, 1997), and PCR ribotyping (McManus and Jones, 1995b). With a small change in the size of the 0.9 kb PCR fragment from plasmid pEA29 of *E. amylovora* strains (Merighi, 1996; Lecomte *et al.*,

1997), individual strains could be placed into groups, but this RFLP corresponded barely to the area where the *E. amylovora* strains were isolated. Application of pulsed-field gel electrophoresis (PFGE) revealed several RFLPs in *E. amylovora* strains from different origins (Zhang and Geider, 1997). The *E. amylovora* strains from Europe and the eastern Mediterranean region were clustered into three groups corresponding to their geographic origins based on the RFLPs of *Xba*I-digested chromosomal DNA. In a recent survey of *E. amylovora* strains from France and England, their PFGE-patterns comprised several groups (S. Jock and K. Geider, unpublished).

In order to find links between the epidemiology of fire-blight in eastern Europe and that of the Mediterranean region, strains isolated at different times and from different geographic areas including Italy were investigated by PFGE analysis of their genomic DNA.

MATERIALS AND METHODS

Bacterial strains. The geographic origins, sources, dates of isolation, and other relevant features of bacterial strains used in the experiments are listed in Table 1. Before this study was initiated, each strain was identified as *E. amylovora* by molecular (Bereswill *et al.*, 1992; 1995) and conventional methods, including HR on tobacco and pathogenicity tests (Schaad, 1988). Furthermore, the closely related PFGE patterns were used as such for identification of *E. amylovora* (Zhang and Geider, 1997).

Table 1. Bacterial strains assayed in the experiments.

Country/Strain	Origin, year of isolation (source or reference)	PFGE type
<i>Albania</i>		
IMB-AL2906	Albania, <i>Pyrus communis</i> , 1995 (Pace and Mazzucchi, 1996)	Pt2
<i>Austria</i>		
295/93	Vorarlberg, Austria, <i>Cotoneaster</i> sp., 1993 (M. Keck)	Pt1
674/94	Vorarlberg, Austria, <i>P. communis</i> , 1994 (M. Keck)	Pt1
<i>Bulgaria</i>		
115.22	Bulgaria, <i>C. oblonga</i> , 1989 (S. Bobev)	Pt5
202.1	Bulgaria, <i>C. oblonga</i> , 1989 (S. Bobev)	Pt2
<i>Croatia</i>		
CR03	Croatia, <i>P. communis</i> , 1996 (B. Cvietkovik)	Pt2
CR04	Croatia, <i>Crataegus</i> sp., 1996 (B. Cvietkovik)	Pt2
CRIV	Croatia, <i>Crataegus</i> sp., 1996 (B. Cvietkovik)	Pt2
<i>Czechia</i>		
BPIC1631	Czechia, <i>Crataegus</i> sp., 1989 (V. Kudela)	Pt1

(continued)

Table 1 (continued)

Country/Strain	Origin, year of isolation (source or reference)	PFGE type
<i>Egypt</i>		
Ea4/82	Egypt, <i>P. communis</i> , 1982 (Falkenstein <i>et al.</i> , 1988)	Pt2
Ea4/83	Egypt, <i>Malus sylvestris</i> , 1983 (Falkenstein <i>et al.</i> , 1988)	Pt2
<i>France</i>		
CFBP 1367	northern France (Lille), <i>Crataegus</i> sp., 1972 (J. P. Paulin)	Pt3
CFBP1430	northern France (Lille), <i>Crataegus</i> sp., 1972 (J. P. Paulin)	Pt3
2650	Ville de Paris, France, <i>Cotoneaster franchettii</i> , 1995 (J.P. Paulin)	Pt3
<i>Germany</i>		
Ea1/79	Germany, <i>Cotoneaster</i> sp., 1979 (Falkenstein <i>et al.</i> , 1988)	Pt1
<i>Greece</i>		
BPIC847	Arkadia, Greece, <i>P. communis</i> , 1984 (P.G. Psallidas)	Pt2
BPIC913	Crete, Greece, <i>P. communis</i> , 1985 (P.G. Psallidas)	Pt2
BPIC928	Mitilini, Greece, <i>P. communis</i> , 1986 (P.G. Psallidas)	Pt2
<i>Hungary</i>		
H895	Nyárlörinc, Hungary, <i>Malus</i> sp. cv. Golden Delicious, May 1996 (J. Németh)	Pt2
H898	Sarkad, Hungary, <i>Pyrus</i> sp., May 1996 (J. Németh)	Pt2
H902	Borota, Hungary, quince, May 1996 (J. Németh)	Pt2
H909	Zákányszék, Hungary, <i>Malus</i> sp. cv. Jonathan, June 1996 (A.N. Kovács)	Pt2
H910	Mohács, Hungary, quince, June 1996 (A.N. Kovács)	Pt2
H931	Mezőkovácsháza, Hungary, medlar, July 1996 (J. Németh)	Pt2
<i>Israel</i>		
Ea209	Israel, <i>Pyrus</i> sp. (S. Manulis)	Pt5
Ea226	Israel (S. Manulis)	Pt5
Ea263	Israel, <i>Cydonia</i> sp. (S. Manulis)	Pt2
Ea267	Israel, <i>Malus</i> sp. (S. Manulis)	Pt2
<i>Italy</i>		
11LE	Lecce, Apulia, Italy, <i>P. communis</i> , 1990 (C. Cariddi)	Pt2
109BA	Bari, Apulia, Italy, <i>P. communis</i> , 1990 (C. Cariddi)	Pt2
OMP-BO786.1/91	Messina, Sicily, Italy, <i>P. communis</i> , 1991 (A. Calzolari)	Pt2
ISPAVE094	Caserta, Campania, <i>P. pyrifolia</i> , 1996 (L. Corazza; Griffo <i>et al.</i> , 1998)	Pt1
OMP-BO691.2/95	Bologna, Emilia-Romagna, Italy, <i>P. communis</i> , 1995 (A. Calzolari)	Pt3
OMP-BO1077.7/94	Bologna, Emilia-Romagna, Italy, <i>P. communis</i> , 1994 (A. Calzolari)	Pt3
OMP-BO1160.2/94	Bologna, Emilia-Romagna, Italy, <i>Crataegus</i> sp., 1994 (A. Calzolari)	Pt3
OMP-BO1178.1A/94	Bologna, Emilia-Romagna, Italy, <i>Sorbus</i> sp., 1994 (A. Calzolari)	Pt3
OMP-BO1204.1/94	Bologna, Emilia-Romagna, Italy, <i>Pyracantha</i> sp., 1994 (A. Calzolari)	Pt3
<i>Spain</i>		
FB2a	Basque Country, Spain, <i>M. baccata</i> , 1995 (M.M.Lopez).	Pt4
<i>Switzerland</i>		
331.93	Neerach, Switzerland, <i>M. communis</i> , 1993 (T. Hasler)	Pt1
345.90	Eschenz, Switzerland, <i>C. salicifolius</i> , 1990 (T. Hasler)	Pt1
508.94	Oberrohrdorf, Switzerland, <i>Crataegus</i> sp., 1994 (T. Hasler)	Pt1

OMP-BO: Osservatorio per le Malattie delle Piante, Bologna, Italy (*i.e.* Regional Phytosanitary Service).

ISPAVE: Istituto Sperimentale per la Patologia Vegetale, Roma, Italy.

CFBP: Collection Française des Bactéries Pathogènes, Angers, France.

BPIC: Benaki Phytopathological Institute Collection, Athens, Greece.

PFGE analysis. The methods used to grow strains, for the preparation of genomic DNA samples and for PFGE analysis were described previously (Zhang and Geider, 1997). Briefly, the cultures were grown to an optical density of 0.6 ($A_{600\text{ nm}}$) or adjusted to this density after centrifugation. Lysis of the agar-embedded cells was for three days, followed by overnight digestion with a restriction enzyme (*Xba*I or *Spe*I). The plugs were embedded in the running gel, which was run with a linear ramping time of 1 s to 25 s for 22 h at 14°C in a chilled BioRad CHEF-DRIII system.

RESULTS

PFGE analysis of *Xba*I digested chromosomal DNA from *E. amylovora* strains. Restriction digests and subsequent PFGE analysis of genomic DNA of *E. amylovora* can result in rare but significant changes of banding positions of DNA fragments. The RFLPs obtained with *Xba*I-digested chromosomal DNA of *E. amylovora* were used to group strains from different geographic regions (Zhang and Geider, 1997). Grouping into the patterns CE, FR, and MT has been refined here with additional PFGE pattern types and the pattern attributed to strains from Western France has been narrowed to strains 2650, CFBP1367, CFBP1430 from the regions of Paris and Lille, called here northern France (Tables 1 and 2). PFGE assays were applied in this study to strains isolated in eastern Europe and the Mediterranean region, and part of the data are shown in Figs 1 and 2. The assays include strains from Israel (4), Greece (3), Albania (1), Bulgaria (2), Hungary (6), Croatia (3), Czechia (1), Austria (2) and Italy (9). Strains from Switzerland (3) and Spain (1) were also included and all were compared to other previously investigated strains originating from central Europe and northern France. The strain FB2a from Spain carried the pattern previously found for a strain from England (Ea775) and not the pattern of strains from northern

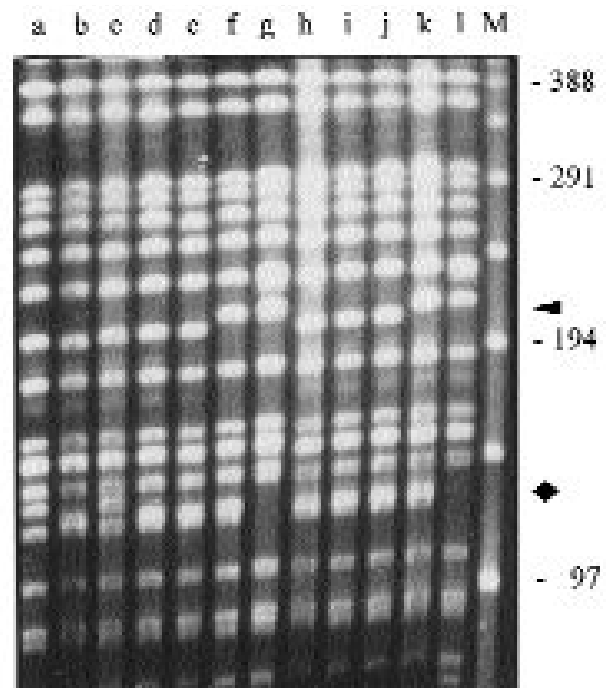


Fig. 1. PFGE-profiles after *Xba*I digests of genomic DNA from *E. amylovora* strains isolated in Israel, Croatia and Italy compared to strains with known PFGE patterns. Lane a-d: Ea209, Ea267, Ea226, Ea263 (Israel); e: Ea4/82 (Egypt); f: Ea1/79 (Germany); g: 2650 (France); h-j: CRO3, CRO4, CRIV (Croatia); k: ISPAVE094 (southwestern Italy); l: OMP-BO691.2 (northeastern Italy); M: molecular weight ladders of phage λ -DNA oligomers (Boehringer Mannheim). Linear ramping time 5 s to 20 s for 18 hours and 1 s to 25 s for 21 hours at 5 V cm^{-1} . An additional band of Ea209 and Ea226 (lanes a and c), indicating the variation in pattern Pt5, is marked by a quadrangle. Bands indicating the PFGE pattern types Pt1/Pt2 are marked by an arrowhead and the lacking band for type Pt3 by a rhombus.

France. To demonstrate stability of PFGE patterns, strain Ea1/79 was transferred 16 times from single colonies on LB agar plates. The observed PFGE pattern after an *Xba*I digest was still identical with that of the parental strain.

Table 2. New scheme for classification of *E. amylovora* strains from Europe and the Mediterranean region.

New PFGE pattern type	Previous classification	Early strain with pattern	Origin	Year	Reference for pattern
Pt1	CE	Ea595	England*	1958	Zhang and Geider,1997
Pt2	MT	Ea4/82	Egypt	1982	Zhang and Geider,1997
Pt3	FR	CFBP1430	France*	1972	Zhang and Geider,1997
Pt4	-	Ea775	England*	1959	Zhang and Geider,1997
Pt5	-	115.22	Bulgaria*	1989	This work

*Other major patterns have been found in the same country.

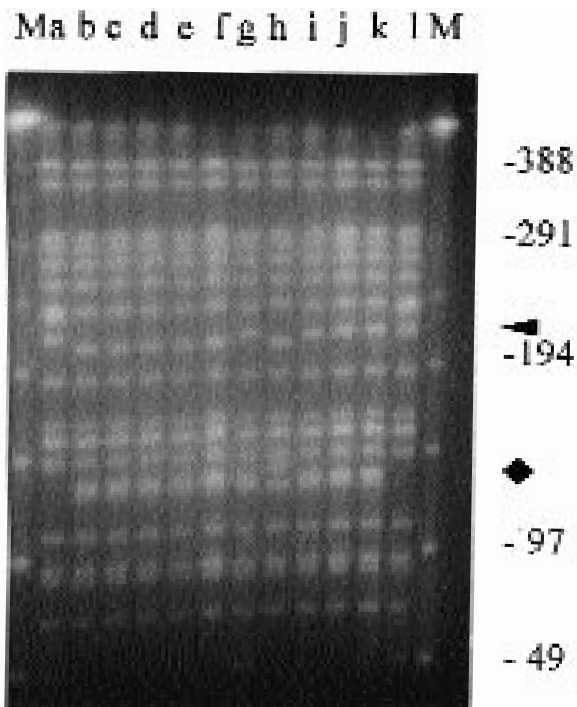


Fig. 2. PFGE-profiles after *Xba*I digests of genomic DNA from *E. amylovora* strains isolated in Italy and eastern European countries compared to strains with known PFGE patterns. Lane a: OMP-BO1077.7 (northeastern Italy); b: 109BA (southern Italy); c: 11LE (southern Italy); d-e: BPIC847, BPIC913 (Greece); f: IMB-AL2906 (Albania); g: BPIC1631 (Czechia); h: 115.22 (Bulgaria); i: Ea4/83 (Egypt); j: 295/93 (Austria); k: Ea1/79 (Germany), l: CFBP1367 (northern France). M: molecular weight ladders of phage λ -DNA oligomers (Boehringer Mannheim). Linear ramping time 5 s to 20 s for 18 hours and 1 s to 25 s for 20 hours. Bands indicating the PFGE pattern types Pt1/Pt2 (arrowhead) and Pt3 (rhombus) are marked.

Patterns of strains from eastern Europe and Israel.

Most strains from other eastern European countries, namely from Albania, Croatia, Greece and Hungary, had a similar PFGE type (Table 1). Three strains, one from Bulgaria (Fig. 2h) and two from Israel (Fig. 1a, c), showed a difference in a band around 135 kb. This change put those strains into another subgroup of strains from the Mediterranean region that was named Pt5. The Pt2 pattern of the other strains assayed from Bulgaria and Israel grouped those together with the strains from Egypt and Turkey, described previously (Zhang and Geider, 1997).

Strain BPIC1631 from Czechia (Fig. 2g) belonged to the central European group, now called Pt1. The strains from Switzerland and from Austria also showed the same pattern (Fig. 2j). This pattern differs from that of Mediterranean strains of type Pt2 by a shift of a 210 kb *Xba*I-fragment to 220 kb.

***E. amylovora* strains isolated in Italy.** Nine strains from different parts of Italy were investigated in this study (Table 1). They had three typical *Xba*I-patterns which were found by Zhang and Geider (1997), *i.e.* pattern Pt2 (previously named MT) found for most strains of the eastern Mediterranean region, the pattern Pt3 (FR), found for strains from northern France, and the pattern Pt1 (CE), found for strains from central Europe. Five strains from northeastern Italy, OMP-BO1077.1/94, OMP-BO1160.2/94, OMP-BO1178.1A/94, OMP-BO1204.1/94, and OMP-BO691.2/95, had the pattern Pt3 (Figs 1, 2 and Table 1), which differs in the lack of *Xba*I-fragments at 130 kb from the pattern Pt1. Three strains isolated from southern Italy showed the Mediterranean pattern Pt2. Only strain ISPAVE094, isolated from the Campania region (southwestern Italy), showed the pattern Pt1 (Table 1). Because *E. amylovora* strains from northern France display a typical *Spe*I pattern with a large, dominant 800-kb fragment (Zhang and Geider, 1997), all the strains from Italy with the pattern Pt3 were also analyzed by digestions with this restriction enzyme. As expected, the strains with Pt3-pattern shared the *Spe*I pattern with strains from northern France (Fig. 3).

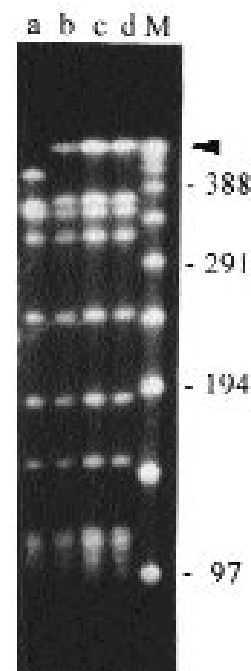


Fig. 3. PFGE-profiles of genomic DNA from various *E. amylovora* strains after *Spe*I digest. Lane a: OMP-BO786.1/91; b: OMP-BO1160.2/94; c: OMP-BO1178.1A/94; d: OMP-BO1204.1/94; M: molecular weight ladders of phage λ -DNA oligomers (Boehringer Mannheim). Linear ramping time 5 s to 20 s for 18 hours and 1 s to 25 s for 20 hours. The typical 800-kb fragment for strains from northern France is marked by an arrowhead.

DISCUSSION

Fire-blight was restricted to North America until this century. The disease was then observed in New Zealand in 1919 and later in England from 1956 onward. Other parts of Europe were hit by the disease in subsequent years, especially areas adjacent to the North Sea. In the Mediterranean region, Egypt was the first country, where fire-blight was reported, and the disease gradually moved north via Israel to Turkey. Later, many eastern European countries were affected such as Greece and its islands. Most recently, fire-blight was reported in Hungary. *E. amylovora* strains isolated from various origins at different times were analyzed by PFGE after digestion with rare cutting restriction enzymes (Zhang and Geider, 1997). In subsequent assays, the dominant Mediterranean pattern Pt2 was not found for a few strains from Israel and Bulgaria with a change in an *Xba*I fragment of genomic DNA, which were grouped as Pt5. This could mean a spontaneous mutation in a strain, although we cannot say if there is any connection between fire-blight outbreaks in these two countries. By PFGE analysis after digestion with *Xba*I, *E. amylovora* strains from Israel are not homogenous in contrast to screening with serological methods and the band patterns obtained from arbitrarily primed PCR (Manulis *et al.*, 1998).

Another wave of the disease has moved from central Europe to the adjacent countries, such as Switzerland, Czechia, and Austria and possibly eastern France. These strains show the pattern Pt1 different for strains from northern France. With the occurrence of fire-blight in Spain further spread from France was assumed. In this study, only one strain from Spain was assayed, producing a pattern different from Pt3. However, more strains from Spain and from other parts of France must be assayed for a possible relationship of strains from these areas. A recent survey of *E. amylovora* strains from many parts of France revealed a diversity of PFGE-patterns among western, northern and eastern France (S. Jock and K. Geider, unpublished).

Recent outbreaks of fire-blight in Italy renewed the question about the possible origin of strains isolated in this country (Fig. 4). The detection of the Pt2-strains from Apulia and Sicily regions (southern Italy) could be explained with the assumption that the disease was introduced from areas of the Mediterranean basin (southeastern Europe-Middle East) by natural vectors (*e.g.* migratory birds, insects, aerosols, etc.). In fact, for these regions an officially documented import of pome-fruit propagation material does not exist (Regional Phytosanitary Service, Bologna, personal communication). The pattern Pt3 shown by the strains isolated in the



Fig. 4. Map of Italy with isolation sites of *E. amylovora* strains assayed by PFGE.

Emilia-Romagna region (northeastern Italy) might support the hypothesis that the way of entry for *E. amylovora* was through latently infected plant propagation material. In this case, trade of such plant material is well documented. Only a few strains with the pattern Pt3 have been found in the PFGE screening. These originated from northern France, but could also occur in adjacent regions and countries. The strain ISPAVE094 was isolated in 1996 from a single young infected tree of Asian pear (*P. pyrifolia*) of an orchard grown in the Campania region (southwestern Italy). The graftlings were originally imported from central Europe by a nursery located in an area of northwestern Italy, still free of fire-blight (L. Corazza, personal communication). Pattern Pt1 shown by this latter strain implies a third introduction of fire-blight into Italy.

The PFGE pattern of *E. amylovora* strains seems to be stable after several transfers in the laboratory, when assayed for strains of a region, independent of changes in other characteristics of strains such as levan production, or the extent of amylovoran synthesis (Zhang and Geider, 1997). Some of our strains may be not representative for areas discussed here such as only one strain from Spain or one strain from the Campania region (southwestern Italy). This is not due to save lab work dealing with the difficult analysis of genomic fragments



Fig. 5. A map for differentiation of *E. amylovora* strains from parts of Europe and the Mediterranean region. Pt1: pattern typical for central Europe; Pt2: for the Mediterranean Region; Pt3: for northern France. The distribution of typical PFGE patterns is also based on previous data (Zhang and Geider, 1997). *: area, where an *E. amylovora* strain with the central European pattern (Pt1) was isolated.

of *E. amylovora* (Zhang and Geider, 1997), but to difficulties in getting access to many strains from all desired areas or to the rare occurrence of the disease in the field, e.g. only one infected tree in the Campania region.

This report intends to differentiate the fire-blight pathogen in several European areas with cultivation of apple and pears (Fig. 5), but cannot cover local specialties of distribution of *E. amylovora*. Hot zones should be overlaps of strains from central Europe with strains from the Mediterranean region between Austria and Hungary, of strains displaying different patterns within France, the occurrence of fire-blight in Spain, and the multiple patterns of strains from Italy. Another question is the frequency of changes in the PFGE pattern of genomic DNA in nature. For all strains analyzed in our laboratory by PFGE assays, only three strains from the Mediterranean region (Pt5) and recently one strain from Germany did not show exactly the pattern found previously for other strains isolated in the same region.

Further detailed work has to be done to possibly understand spread of *E. amylovora* and to describe the ways of local distribution.

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