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Fire blight (*Erwinia amylovora* [Burrill] Winslow) in Morocco: importance, geographical distribution and characterization

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Summary. Fire blight, caused by *Erwinia amylovora*, was detected in Morocco in 2006 and has spread rapidly throughout the most important pome fruit-producing regions. Surveys were carried out in 2009 in the main pome fruit-growing areas in Morocco to evaluate the current situation of the disease in the country, particularly in the El Hajeb region, where important losses due to this pathogen were recorded. Samples showing symptoms associated witho the disease were collected from affected apple (*Malus domestica*), pear (*Pyrus communis*) and quince (*Cydonia oblonga*) trees and processed for the isolation and purification of the causal agent. Other isolates collected in the period 2006–2008 were also included in this study. All the isolates were identified to genus and species levels using morphological, biochemical and serological tests. Confirmation tests were carried out using classical PCR and Real-time PCR. Forty eight Moroccan isolates were confirmed to belong to *E. amylovora*. Fingerprinting methods (rep-PCR and fAFLP) showed a diversity of the isolates and resulted in grouping them in four separate subgroups. This study suggests that Moroccan isolates of *E. amylovora* have multiple geographical origins.

Key words: fingerprinting, Rep-PCR, fAFLP.

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) (Winslow *et al*., 1920) is a very serious and destructive disease of pome fruits and many ornamental plants from the *Rosaceae* family (Ordax *et al*., 2006). This disease has been reported in more than 46 countries around the world (Van der Zwet, 2006). Consequently, there has been growing concern about eradication measures and detection techniques to reduce the dissemination of the pathogen (Llop *et al*., 2006). The disease was first reported by Denning in 1794 from the North American continent in the upper Hudson River Valley of New York. In 1900, fire blight was identified in Ontario, Canada and reached the western states of California, Oregon and Washington. The disease was then reported in Japan in 1903, New Zealand in 1919, Bermuda in 1938 and Mexico in 1943. It spread eastward and was declared in England in 1957 and in Egypt in 1964. From those two locations the disease spread rapidly through Western Europe and the Middle East regions, respectively. About 45 years later, fire blight was reported from all countries in Europe (Van der Zwet, 2002).

Fire blight has been responsible for serious economical losses in countries where it has oc-

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curred and particularly on the hosts pear, apple, and quince. In 1976, a loss of 4.7 million dollars, mainly in pears, was reported in California, USA. In 1991 a severe outbreak in South-Western Michigan resulted in an estimated loss of 3.8 million dollars (Smith and Lattimore, 1997). In Egypt, by 1988 fire blight destroyed 80% of all pear acreage leading to eradication of 50% of all trees (Bonn and Van der Zwet, 2000). More recently, fire blight caused losses of 68 million dollars in North-West America, 10 million dollars in one region of New Zealand, and 500,000 trees were destroyed in Lebanon and in Italy (Emilia-Romagna) (Vanneste, 2000). Since the discovery of fire blight in Morocco in May 2006 (Fatmi *et al.,* 2008), the disease spread to most of the pome fruit producing regions, inducing severe damage.

Erwinia amylovora is a genetically homogeneous species (Momol and Aldwinckle, 2000), and up till now no characteristics have been found that can clearly distinguish strains by geographical origin, original host or year of isolation. This homogeneity has been confirmed with biochemical, serological and host range studies (Vantomme *et al*., 1982, 1986; Momol and Aldwinckle, 2000). The exceptions are the strains isolated from *Rubus* spp., which show a distinct host range (Beer *et al*., 1996) and could be distinguished from strains of the *Maloideae* group by fatty acid analysis (Van der Zwet and Wells, 1993) and by genetic fingerprinting (Maes *et al*., 1996; Maes and Crepel, 1997; Momol *et al*., 1997, 1999). *Erwinia* strains isolated from pears in Japan can also be distinguished from the *Maloideae* group by pathogenic, biochemical and genetic characteristics. These strains have been described as *E. pyrifoliae* (Rhim *et al.,* 1999), but their taxonomical position is not yet clear (Momol and Aldwinckle 2000; Kim *et al*., 2001). Roselló *et al*., (2008) also described the deviating *E. amylovora* strains isolated from necrotic pear blossoms in Valencia, Spain. By phylogenetic analysis of 16S rDNA, such strains could not be robustly grouped with any single known species (*E. amylovora*, *E. pyrifoliae*) and consequently they were proposed as members of a new species for which the name *Erwinia piriflorinigrans* was proposed. The pulse-field gel electrophoresis (PFGE) analysis of genomic DNA after *Xba*I digestion has revealed six genomic patterns for European strains of *E. amylovora*, providing useful information for strain differentiation, and has been used to analyze the spread of fire blight in Europe (Jock *et al*., 2002).

Some correlation between the profiles of *E. amylovora* strains and the origin of the strains was revealed using the restriction fragment length polymorphism analysis (RFLP) (Atanasova *et al*., 2009).

An objective of the present study was to evaluate a technique that could explore the complete genome, and reveal additional differences, within the *E. amylovora* population present in Morocco. Fluorescent amplified fragment length polymorphism (fAFLP) analysis is a high resolution technique for fingerprinting of bacteria, including plant pathogens (Janssen *et al*., 1996; Rademaker *et al*., 2000; Cirvilleri *et al*., 2006; Cirvilleri *et al.,* 2007; Scuderi *et al*., 2009). The technique has proved to be comparable with DNA:DNA hybridization pairing studies and RFLPs when genetic relatedness or intrapathovar diversity is examined, and has shown more discriminatory power than random amplified polymorphic DNA (RAPD) or PCR using repetitive element primers, so called rep-PCR (Clerc *et al*., 1998; Restrepo *et al*., 1999). Furthermore, Donat *et al*. (2007) reported AFLP analysis as the most sensitive technique currently available to distinguish *E. amylovora* strains, resulting in the discrimination of 13 different groups of Spanish strains that were related to geographical origin.

The aim of the present study was to collect, identify and characterize *Erwinia amylovora* isolates from Morocco in comparison with some reference strains from different countries, and to determine their variability and the current situation of fire blight disease in this country.

Materials and methods

Field survey, collection of diseased samples and bacterial strains used

Plant material was collected from 2006 to 2009. Surveys were made in the most important pome fruit production regions of Morocco (Meknes, El Hajeb, Sefrou) during early and late spring each year according to the development of fire blight. During the visits, plant samples (twigs, branches, flowers, leaves, fruits) from plants in diseased orchards were taken, labelled, stored in plastic bags

and brought to the Phytobactériology laboratory at the Institut Agronomique et Vétérinaire Hassan II, Complexe Horticole d'Agadir for isolation. The geographical origin, host, variety and date of isolation from the samples are given in Table 1. After isolation and purification (see below), the isolates were maintained on YDC and NBY slants in screw-capped tubes at 4°C.

Some reference strains obtained from Canada, Denmark, Egypt, Italy, New Zealand, United Kingdom and United States of America were added for comparison and reference purposes. Bacterial strains used in this study are listed in Table 1.

Isolation and purification

Diseased samples were washed in tap water. Infected tissues were surface sterilized by immersion in 10% household bleach for 3 min and rinsed twice in sterile distilled water (SDW) for a few minutes. In the case of flowers or leaves, the samples were each macerated in a few drops of SDW in a sterile glass Petri dish using a sterile scalpel and forceps. Thirty minutes after maceration, 30 µL of macerated tissue were streaked onto King's medium agar B (KB) (King *et al*., 1954). In the case of branches, they were cut into disc pieces and these pieces were placed on water agar (10%). The plates were then incubated at 27°C for 2–3 days and observed daily for bacterial growth. Suspected colonies of *E. amylovora* (white, circular, mucoid, and curved) were selected and further purified on KB agar at 27°C. This operation was repeated three to four times to be sure that pure cultures were obtained for identification tests (Jones and Geider, 2001).

For the identification of the obtained isolates to the species level, physiological and biochemical, pathogenicity, immunofluorescence, PCR, and Real-time PCR tests were used. Forty eight Moroccan isolates and seven reference strains of *E. amylovora* (LMG 1877, LMG 2022, LMG 2024^T, LMG 2067, LMG 2074, 40035, and 40062) were included in these tests (Table 1).

Physiological and biochemical identification

To characterize the isolates to genus level, the following key tests were performed: Gram reaction; colony morphology on King's B medium (KB), yeast dextrose calcium carbonate medium (YDC) (Kaneishiro *et al*., 2008) and semi selective CCT medium (Ishimaru and Klos, 1984); fermentative metabolism of glucose; tobacco hypersensitive reaction; potato rot; oxidase and catalase tests. All the reference strains were included in these tests.

For characterization to species level, gelatin hydrolysis; levan production; nitrate reduction; citrate; urease; indole production; growth at 36°C and at 39°C; acid production from salicin, inositol, L-arabinose and sorbitol were performed according to Jones and Geider (2001).

Pathogenicity

A pathogenicity test was carried out for all tested strains (Table 1) as described by EPPO (EPPO, 2004). Briefly, a loopful of a 48 h-old culture of each isolate, previously grown on KB medium, was used to prepare a bacterial suspension in SDW of about 10^8 cfu mL^{-1}. Leaves from healthy pear plants (cv. Doc Guyot) were first washed in tap water and surface sterilized by immersion in a solution containing 10% of household bleach for 30 seconds. The leaves were then rinsed three times successively in SDW and placed in sterile glass Petri dishes. The inoculum of each isolate was introduced into the leaves by making a wound along the principal vein using a scalpel previously impregnated in the corresponding bacterial suspension. Inoculated leaves were placed separately in tubes containing sterile 1% water agar by introducing the petiole in the medium. The tubes were kept in a humid chamber at 20–25°C and observed daily for a week. Three leaves were used for each isolate. For negative controls, SDW was used.

Serological test

The identity of the isolates was also checked using indirect immunofluorescence according to the method described by Van de Bilt *et al*. (2008). Two primary antibodies against *E. amylovora* were used separately: one commercial polyclonal antibody (IVIA EPS 1430, Plant Print Diagnostics, Valencia, Spain) was used at the dilution of 1/200 in phosphate buffer saline (PBS). The second was created in the phytobacteriology laboratory (Agadir, Morocco) in 2007 using strain EaMr01-06 and was used at the dilution of 1/200. The fluorescent antibody (Anti-rabbit IgG, FITC conjugate, SIGMA, St. Louis, MO, USA) was used at the dilution of 1/80 in PBS. For negative controls, PBS was used instead of bacterial suspension.

Table 1. List of *Erwinia amylovora* isolates from Morocco and reference bacterial strains used in this study.

Isolate	Host		Origin, year of isolation
	Species	Variety	
Erwinia amylovora isolates from Morocco			
EaMr01-06, EaMr06-06, EaMr07-06	Pear	Williams	Meknes, 2006
EaMr13-06, EaMr14-06, EaMr18-06	Pear	P-Crassane	Meknes, 2006
EaMr19-06, EaMr20-06, EaMr21-06	Quince	Champion	Meknes, 2006
EaMr28-06, EaMr29-06, EaMr32-06, EaMr33-06, EaMr35-06, EaMr36-06	Apple	Galla-Rev	Meknes, 2006
EaMr41-06, EaMr42-06 EaMr44-06,	Quince	Vranja	Meknes, 2006
EaMr54-08, EaMr55-08, EaMr62-08, EaMr63-08, EaMr64-08, EaMr71-08, EaMr72-08, EaMr73-08	Pear	P-Crassane	Meknes, 2008
EaMr56-08, EaMr57-08	Pear	Williams	Meknes, 2008
EaMr60-08, EaMr61-08, EaMr68-08, EaMr69-08, EaMr70-08	Pear	Doc Guyot	Meknes, 2008
EaMr65-08, EaMr66-08, EaMr67-08	Pear	Goscia	Meknes, 2008
EaMr74-08, EaMr75-08, EaMr76-08, EaMr77-08,	Pear	Doc Guyot	Sefrou, 2008
EaMr78-09, EaMr79-09, EaMr80-09	Pear	Doc Guyot	Sefrou, 2009
EaMr81-09	Apple	Start Krinson	El Hajeb, 2009
EaMr82-09	Apple	Golden	El Hajeb, 2009
EaMr83-09	Pear	P-Crassane	El Hajeb, 2009
EaMr84-09	Pear	Doc Guyot	El Hajeb, 2009
EaMr85-09	Quince	Champion	El Hajeb, 2009
Reference strains of *Erwinia amylovora*			
LMG 1877	Quince		Denmark, 1972
LMG 2022 (NCPPB 311)	Pear		Canada, 1952
LMG 2024 [T] (ATCC 15580)	Pear		United Kingdom, 1971
LMG 2067 (NCPPB 1735)	Pear		Egypt, 1965
LMG 2074 (NCPPB 2080)	Pear		New Zealand, 1968
40035 (ATCC 15357 [T])	Apple		USA, 1949
40062	Pear		California, 1971
EaI 204	Pear		Italy, 2002
EaI 205	Pear		Italy, 2002
EaI 208	Pear		Italy, 2002
Other bacterial species			
Pectobacterium carotovorum subsp. *carotovorum* (LMG 2408)	Lily of the Nile		Denmark, 1955
Pectobacterium carotovorum subsp. *carotovorum* (UNIBA 17)	Potato		Italy, 1998
Pectobacterium atrosepticum (UNIBA 16)			Italy, 1995
Dickeya chrysanthemi (UNIBA 18)			Italy, 1997
Pseudomonas syringae pv. *syringae* (PD 1818)	Pear		Netherlands, 1993
Pseudomonas savastanoi pv. *savastanoi* (CFBP 1670[T])	Olive		Yugoslavia, 1959

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; LMG, Laboratorium voor Microbiologie Universiteit Gent, Belgium; UNIBA, University of Bari, Italy; NCPPB, National Collection of Plant Pathogenic Bacteria, UK; PD, Department of Bacteriology, Plant Protection Service, Wageningen, The Netherlands; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France
[b] Supplied by Marco Scortichini, CRA, Rome, Italy.
[T] Type strain.

Polymerase Chain Reaction (PCR)

DNA extraction was done according to Llop *et al*. (1999). PCR was performed with extracted DNA of the isolates and using two specific primers A (5'-CGG TTT TTA ACG CTG GG-3') and B (5'-GGG CAA ATA CTC GGA TT-3'). Both primers are 17-mer oligonucleotides that were obtained from the borders of the pEA29 fragment (Bereswill *et al*., 1992). The PCR was carried out in a total volume of 50 µL: 45 µL of PCR mixture plus 5 µL of extracted DNA of an isolate for each reaction in a thermocycler (GeneAmp PCR system 9600, Perkin Elmer, Norwalk, CT, USA) using the following program: denaturation at 93°C for 5 min followed by 35 cycles of 93°C for 30 s, 52°C for 30 s and 72°C for 75 s. A final step of 72°C for 10 min ended the amplification reaction (Bereswill *et al*., 1992).

The PCR products were separated on a 1.5% agarose, stained with ethidium bromide and photographed under UV light following the method described by EPPO (2004).

Real time - PCR

Primer pair Ea-lscF (5'-CGCTAACAGCAGATCGCA-3') and Ea-lscR (5'- AAATACGCGCACGACCAT-3'), producing a 105 bp amplicons, and the TaqMan probe Ea-lscP (5'-CTGATAATCCGCAATTCCAGGATG-3') used in this study were based on the sequence of the levan sucrose gene (*lsc*, Genbank file X75079) (Lehman *et al*., 2008). The reactions were run in a SmartCycler (Cepheid, Sunnyvale, CA, USA) in 25 µL volumes using for each reaction: 1.5 µL of Dream Taq polymerase (5U μL^{-1}) (Fermentas, Italy); 2.5 µL of buffer 10×; 2.5 µL of $MgCl_2$ (25 mM); 1 µL of dNTPs (10 mM); 1 µL of each primer (25 µM); 0.5 µL of the probe (10 µM); 13 µL of purified water. Two µL of the bacterial suspension of each isolate was added in one reaction. Positive (reference strains) and negative controls (purified water) were included. The cycling conditions consisted of a denaturation 95°C for 5 min for the first step followed by 40 cycles of 95°C for 10 s and 60°C for 16 s.

Fingerprinting techniques

rep-PCR

Bacterial cultures (1.5 mL) grown overnight in Luria Bertani broth (LB) at 27°C were centrifuged at 13.000 *g* for 2 min and the supernatant was discarded. Whole genome DNA extraction was performed by using the GenElute Bacterial Genomic DNA Kit (Sigma), following the manufacturer's recommendations. According to Versalovic *et al*. (1991), PCR reactions were carried out in a 25 µL volume for each and the same protocol was used for both PCR reactions using primers BOX (5'-CTAGGCAAGGCGACGCGCTGACG-3'), for BOX-PCR, and ERIC-f (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-r (5'- AAGTAAGTGACTGGGGTGAGC-3') for ERIC-PCR.

PCR amplifications were performed in a thermoycler (GeneAmp PCR system 9600, Applied Biosystem, Lincoln Centre Drive Foster City, CA, USA) programmed for 2 min at 95°C as preheating, followed by 35 cycles of 30 s at 94°C, 30 s at 92°C and 1 min at 50°C. A final extension at 65°C for 8 min ended the reactions. A negative control to detect reagent contamination was included in each PCR, containing all components except the DNA extract, which was replaced by 1 µl of SDW. Rep-PCR profiles were electrophoresced in the same lane on each gel to allow a direct comparison by visual inspection. For each isolate the bands were scored as (1) and (0) depending on whether or not the isolate did or did not generate a band, respectively. Fingerprints were considered to be highly similar when all visible bands had the same apparent migration distance. Variations in the intensity or shape of bands were not taken into account.

Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) using the Jaccard's coefficient (PHYLIP 3.6 software package), and the robustness of the tree was assessed by bootstrap analysis (1,000 repeated samplings) (Felsenstein, 1985).

fAFLP analysis

Bacterial genomic DNA (250 ng) of each isolate was digested for 4 h at 37°C with *Eco*RI (10 U) and *Mse*I (10 U) enzymes (Invitrogen-Life Technologies, Paisley, UK) in a final restriction volume of 25 µL containing 10× of reaction buffer.

Restriction enzymes were heat-inactivated at 75°C for 15 min and 5 µL of *Eco*RI and *Mse*I adaptors were added to final concentrations of 2 and 20 µM, respectively. They were then ligated for 2 h at 20°C using 2 U of T4 DNA ligase (Invitrogen-

Life Technologies) in a 5× ligase buffer, in a final ligation mixture of 50 µL volume. PCR mixtures (50 µL) consisted of 5 µL of ligated DNA (dilution 1:20 with SDW), 1 µL of 5 µM of each *Mse*I+C and *Eco*RI+A primers (Cirvilleri *et al*., 2006), 1 µL of deoxynucleoside triphosphates dNTPs (10 mM), and 5 U of Taq polymerase (Invitrogen-Life Technologies).

Both primers *Mse*I and *Eco*RI were used with one selective base, C and A respectively, in order to increase the chances of detecting polymorphisms and *Eco*RI+A primer was labeled at the 5' end with cy5 dye (MWG Biotech AG, Ebersberg, Germany). All PCR reactions were performed in a GeneAmp PCR system 9600 thermoycler as following: 60 s at 94°C, 30 s at 65°C, and 60 s at 72°C for one cycle; 12 cycles touch down PCR with annealing temperature reduced from 65°C by 0.7 °C at each cycle; and 25 cycles of 30 s of denaturation at 94°C, 60 s of annealing at 56°C, and 60 s of extension at 72°C. This "touchdown" PCR protocol was used to minimize PCR artifacts (Kassama *et al*., 2002; Cirvilleri *et al*., 2006).

The fAFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA sequencer (Beckman & Coulter, Fullerton, CA, USA). A mixture containing 2 µL of PCR products (diluted at 1:20 in SLS) with 30 µL of SLS (sample loading solution), 0.5 µL of CEQ DNA size standard Kit-600 (used to normalize the profile) and one drop of mineral oil were loaded on a capillary electrophoresis system CEQ 8000. The set-up of the CEQ 8000 was done according to the manufacturer's instructions.

The data, displayed as peaks in electropherogram files, were analyzed using the CEQ 8000 analysis software. The fragment sizes were determined by comparison with the internal DNA Size Standard Kit. Electropherograms of all fAFLP profiles were visually inspected for polymorphisms, with the presence (1) or absence (0) of fragments from 60 to 700 bp scored in a binary matrix and stored in Microsoft Excel.

Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) using the Jaccard's coefficient (PHYLIP 3.6 software package), and the robustness of the tree was assessed by bootstrap analysis (1,000 repeated samplings) (Felsenstein, 1985).

Results

Importance and geographical distribution of fire blight

In the year of its detection (October 2006) in the Meknes region of Morocco, approximately 40 ha of pear consisting of varieties Passe-Crassane, Williams, Doc Guyot, Tosca, Cossia, Morettini, Beurre Hardy and 2 ha including apple (Galla-Rev) and quince (Champion, Vranja) were affected by fire blight disease. Trees in these areas were then uprooted. The losses were estimated to about 4 million Dirhams (approx. 360,000 €). In 2007, the disease occurred in four new foci in the same region (Meknes) affecting a total area of 69 ha, of which 9 ha were uprooted whereas the rest were sanitized (Plant Protection Service of Meknes, Morocco). In 2008, fire blight was detected in 54 orchards in different provinces: Meknes (three orchards), El Hajeb (30), Sefrou (nine), Ifrane (ten), Taounate (one) and Khenifra (one). The total area affected by this disease was estimated to be 620 ha, and 53.5 ha were uprooted (Fatmi, 2009). In 2009, the disease was detected in 42 orchards in El Hajeb and ten orchards in Meknes. The total area affected by this disease was estimated to about 543 ha and 30 ha of trees were uprooted (Table 2).

Physiological and biochemical identification

The tested isolates showed typical morphological colonies on the media used. After three days of incubation, the colonies were 3–4 mm in diameter, mucoid with shiny surfaces, semitransparent and slightly violet on CCT medium; 4–5 mm in diameter, creamy white, circular, convex on YDC medium and 1.5–2 mm in diameter, white, circular, slightly convex with smooth surface and showed no fluorescence under ultraviolet light on KB medium. All isolates were Gram negative and rod shaped, glucose fermentative, catalase and tobacco hypersensitivity positive and oxidase and potato rot negative (Table 3). According to these tests and the results obtained with the reference strains of *E. amylovora*, all Moroccan isolates belonged to the genus *Erwinia* (Holt, 1994).

The data obtained from further tests showed that all the isolates and the reference strains hydrolyzed gelatin, produced whitish and highly mucous colonies on levan medium, were unable to reduce nitrate to nitrite and were negative for urease and indole production (except EaMr76-08,

Table 2. Current situation of fire blight in El Hajeb and Meknes regions in Morocco (Plant Protection Service of Meknes and El Hajeb, 2009).

Province	County	Contaminated orchards (No.)	Total area (ha)	Host	Uprooted area (ha)
El Hajeb	Ait Harzallah	8	140	Pear, apple, quince	62
	Ait Boubimdane	6	58	Pear, apple, quince	9
	Ait Yazem	5	19	Pear, apple	-
	Ait Naamane	2	18	Pear	-
	Ait Bourazouine	4	33	Pear, apple	4
	Sbaae Ayoune	2	23	Pear	-
	Laqsir	11	122	Pear, apple, quince	6
	Tamchachat	4	42	Pear, apple	2
Meknes	Aïn Orma	1	38	Pear, apple, quince	20
	Dar Oum Sultane	1	10	Pear	2
	Aït Walal	2	7.4	Pear	1
	Hamrya	1	3	Pear	2.1
	Ouislane	1	2	Pear	0
	Sidi Slimane Moulkifame	3	24.5	Pear	4.5
	N'Zala Beni Amar	1	3	Pear	0
Total		52	542.9		29.6

which was positive for indole production). All isolates including the reference strains were positive for citrate. No isolate and no reference strains grew at 36°C or at 39°C. They were unable to use inositol and salicin but were able to use sorbitol and L-arabinose. LMG 2022, LMG 2024 and LMG 2074 reference strains did not produce acid from L-arabinose.

Pathogenicity

All isolates and reference strains produced progressive disease symptoms of fire blight on inoculated pear leaves. These leaves showed necrosis that started in the leaf wounds and progressed via the veins into the petioles, and droplets of ooze developed on the leaves. No symptoms were recorded in the case of leaves inoculated with SDW (Figure 1).

Serological test

All the tested isolates reacted positively in indirect immunofluorescence using both the commercial polyclonal antibody and the serum created against *E. amylovora* strain EaMr01-06. All the above mentioned tests identified all Moroccan isolates as *E. amylovora*. No reaction was observed in the case of PBS used as negative control.

Polymerase Chain Reaction (PCR)

The majority of Moroccan isolates (83.3%) and reference strains amplified a band of 1.000 bp using primers A and B with the exception of eight Moroccan isolates (EaMr14-06, EaMr18-06, EaMr20-06, EaMr21-06, EaMr36-06, EaMr42-06, EaMr44-06, EaMr79-09) and one reference strain (40062) which did not show any signal (Figure 2).

Real-time PCR

All Moroccan isolates and reference strains gave positive reactions in real-time PCR using two primers (Ea-lscF/Ea-lscR) and the TaqMan probe (Ea-lscP) designed on levan sucrose gene. The Ct. values ranged from 17.63 to 21.79 with a mean of 18.54 and from 18.57 to 20.90 for the 48-Moroccan isolates and the reference strains, respectively. The Ct. values for the negative control were above 32.28 (Table 4).

Figure 1. Pathogenicity induced by the inoculation of the tested isolates of *Erwinia amylovora* on pear leaves. Left, leaf inoculated with sterile distilled water (control). Right, symptoms and bacterial oozes on inoculated leaf.

Relatedness among strains of *Erwinia amylovora* isolated from Morocco

rep-PCR

The rep-PCR was conducted using BOX and ERIC primers. Banding patterns of *E. amylovora* isolates obtained from different locations in Morocco and reference strains from Canada, Egypt, New Zealand, United Kingdom, Italy, Denmark and USA were all similar and different from patterns of outgroup bacteria.

The fingerprinting with primer BOX generated patterns of 12 major bands between 500 bp and 4000 bp, four of which were polymorphic and used for comparison analysis (Figure 3). The dendrogram shows two major clusters (A and B) based on 91.4% of similarity. In cluster A, *E. amylovora* strains from USA, Canada, UK, Egypt, New Zealand, Denmark, Italy and nine Moroccan isolates from 2006 (EaMr20; EaMr21; EaMr28), 2008 (EaMr74; EaMr75; EaMr77) and 2009 (EaMr78; EaMr80; EaMr85) all formed a lineage with statistical support (91.4% cut off). The remaining 29

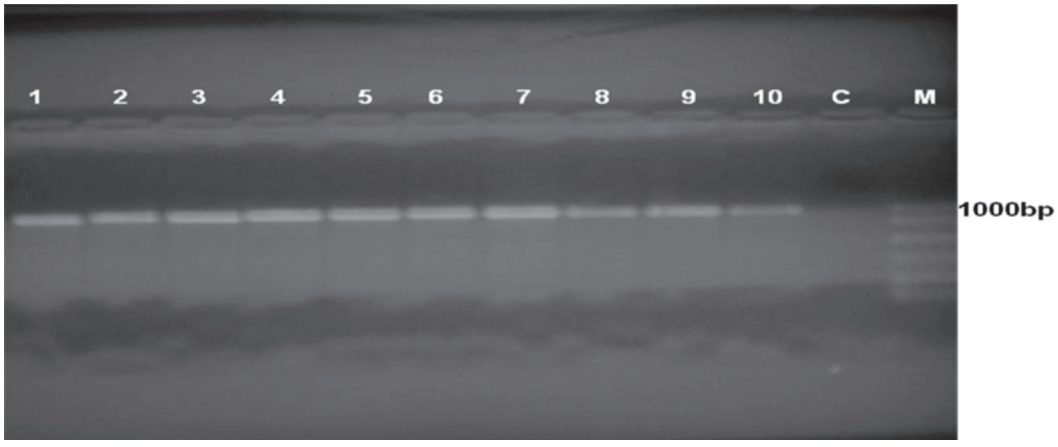

Figure 2. Agarose gel electrophoresis showing a band pattern (1 kb) of PCR amplified product with primers A and B. Lane 1–10, EaMr82-09, EaMr83-09, EaMr84-09, EaMr85-09, LMG1877, LMG 2022, LMG 2024, LMG 2067, LMG 2074, and 40035; lane C, negative control; lane M, molecular marker.

Table 3. Characterization of Moroccan isolates obtained from fire blight diseased plants from 2006 to 2009 and belonging to the genus *Erwinia*.

Isolates	Origin	Gram reaction	White colonies on YDC	Fluorescent pigment on KB	Tobacco HR	Facultative anaerobic growth	Oxidase	Catalase
EaMr isolates[a]	Morocco	-	+	-	+	+	-	+
LMG 1877	Denmark	-	+	-	+	+	-	+
LMG 2022	Canada	-	+	-	+	+	-	+
LMG 2024	UK	-	+	-	+	+	-	+
LMG 2067	Egypt	-	+	-	+	+	-	+
LMG 2074	New Zealand	-	+	-	+	+	-	+
40035 (ATCC 15357)	USA	-	+	-	+	+	-	+
40062	USA	-	+	-	+	+	-	+

[a] Forty-eight isolates from different Moroccan regions that have been identified in this work as *E. amylovora*.

Table 4. Identification of the *Erwinia amylovora* Moroccan isolates by Real time-PCR.

Isolates	Real Time-PCR	
	Ct. values	Results
EaMr strains[a]	17.63<Ct<21.79	+
Control strains[b]	18.57 < Ct <20.90	+
Negative control	Ct > 32.28	-

[a] Forty-eight *Erwinia amylovora* isolates isolated in Morocco between 2006 and 2009.
[b] References *Erwinia amylovora* strains.

Moroccan *E. amylovora* isolates were placed into cluster B. The Moroccan isolate EaMr73-08 and outgroup bacteria were placed outside of these two clusters.

The genomic ERIC-PCR profiles consisted of ten bands ranging in size from 750 bp to 4000 bp, three of which were polymorphic bands. UPGMA fingerprint analysis was performed using the dissimilarity matrix obtained by Jaccard's coefficient. The obtained dendrogram (Figure 4) revealed two distinct cluster groups (E and F) with an overall similarity of about 90%. Eleven *E. amylovora* isolates were grouped in the cluster E, comprising strains from USA, Canada, UK, Egypt, New Zealand, Denmark and four Moroccan isolates from 2009 (EaMr80, EaMr82, EaMr84, EaMr85), whereas in cluster F most of isolates from Morocco (37 Moroccan isolates) and the three Italian strains were grouped together. Outgroup bacteria *Pseudomonas syringae* pv. *syringae* (PD 1818), *Pseudomonas savastanoi* pv. *savastanoi* (CFBP 1670^T), *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2408), *Pectobacterium carotovorum* subsp. *carotovorum* (UNIBA 17), *Pectobacterium atroseptica* (UNIBA 16), *Dickeya chrysanthemi* (UNIBA 18), were placed outside of these two main clusters.

fAFLP

In this study, *f*AFLP analysis was performed with the CEQ 8000 Genetic Analysis System automated DNA sequencer. The results showed the presence of different profiles for the 45 *E. amylovora* strains and four strains from outgroup bacteria.

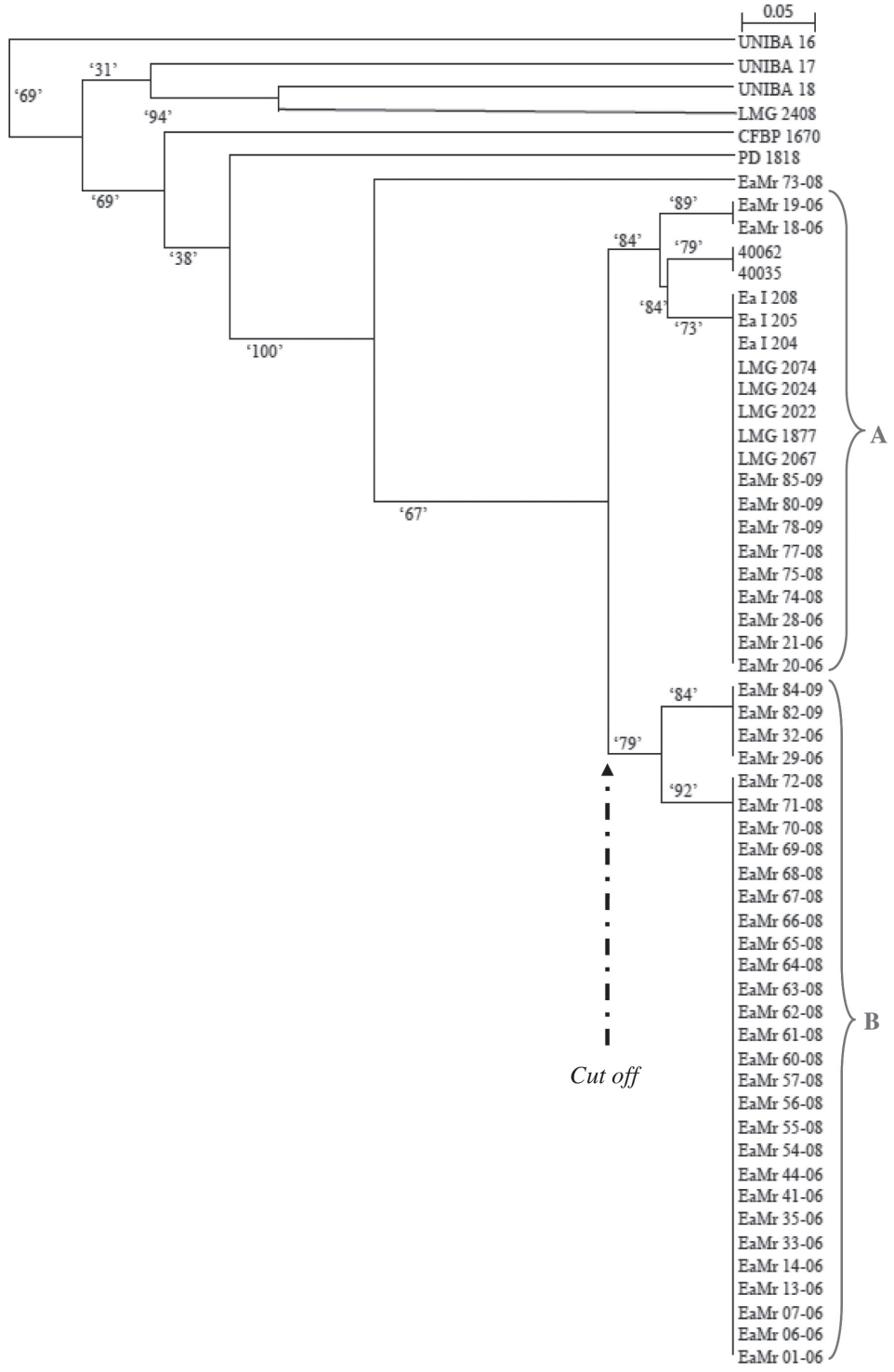

Figure 3. Dendrogram of *Erwinia amylovora* strains derived from rep-PCR DNA fingerprints generated from the BOXA1R primer. Dendrogram was calculated with unweighted pair-group method with average linkages (UPGMA) using the Jaccard's coefficient and the robustness of the tree was assessed by bootstrap analysis.

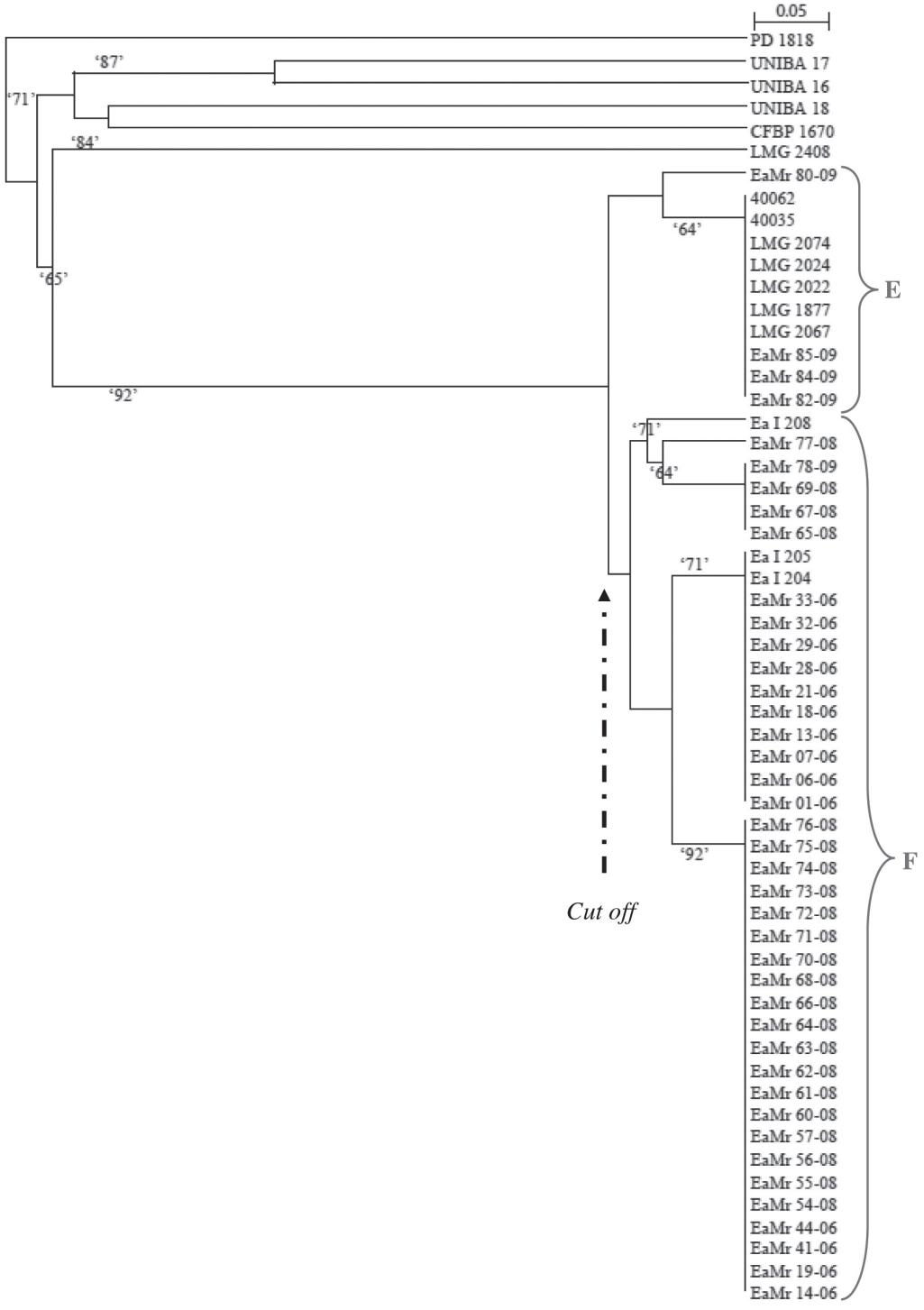

Figure 4. Dendrogram of *Erwinia amylovora* strains derived from rep-PCR DNA fingerprints generated from the ERIC primer. Dendrogram was calculated with unweighted pair-group method with average linkages (UPGMA) using the Jaccard's coefficient and the robustness of the tree was assessed by bootstrap analysis.

The analysis generated 43 to 82 distinctive fragments (15 of which resulted polymorphic bands) upon amplification with the primers *Mse*I+C and *Eco*RI+A. All generated bands (displayed as peaks) were used to compute a similarity matrix with Jaccard coefficient and to perform a cluster analysis. Examples of representative electropherograms are shown in Figure 5. Peak height indicates the relative fluorescence of the detected fragments, and this height did not vary relevantly between replicate runs. The resultant UPGMA dendrogram (Figure 6) showed four main clusters clearly distinguished using 19% of dissimilarity as a cut-off point. Cluster G1 comprised six Moroccan isolates, five of which are from 2006 and one from 2008. All isolates of this group were from Meknes and were isolated from pear. Cluster G2 includes 12 strains from various origins, hosts and years of isolation: seven isolates were from Morocco and five strains from Canada, Egypt, New Zealand, USA and Denmark. In cluster G3 nine strains were grouped, seven of which were from Morocco (two isolates in 2006, four isolates in 2008, one isolate in 2009), one from United Kingdom (LMG 2024) and one from USA (40035). Cluster G4 includes nine Moroccan isolates which were all from Meknes, two from 2006 and seven from 2008. Outgroup bacteria (PD 1818, CFBP 1670, UNIBA 17, UNIBA 16), the Italian strain EaI208, and five Moroccan strains (EaMr19-06, EaMr20-06, EaMr32-06, EaMr57-08, EaMr79-09) were placed outside of these four main clusters, sharing with them only a modest similarity (56%). No correlations were found between the isolates and the host plant, year of isolation and country from where they were originally isolated.

Discussion

After the first discovery of fire blight in Morocco in 2006 and its rapid spread within the most important regions of pome fruit production during 2008 and 2009, the appearance of the quarantine bacterium *E. amylovora* may be expected to reach the other pome fruits producing regions of the country in the future. The implementation of long-term management of fire blight is essential. This requires appropriate strategies and involvement of all people dealing with the pome fruit in-

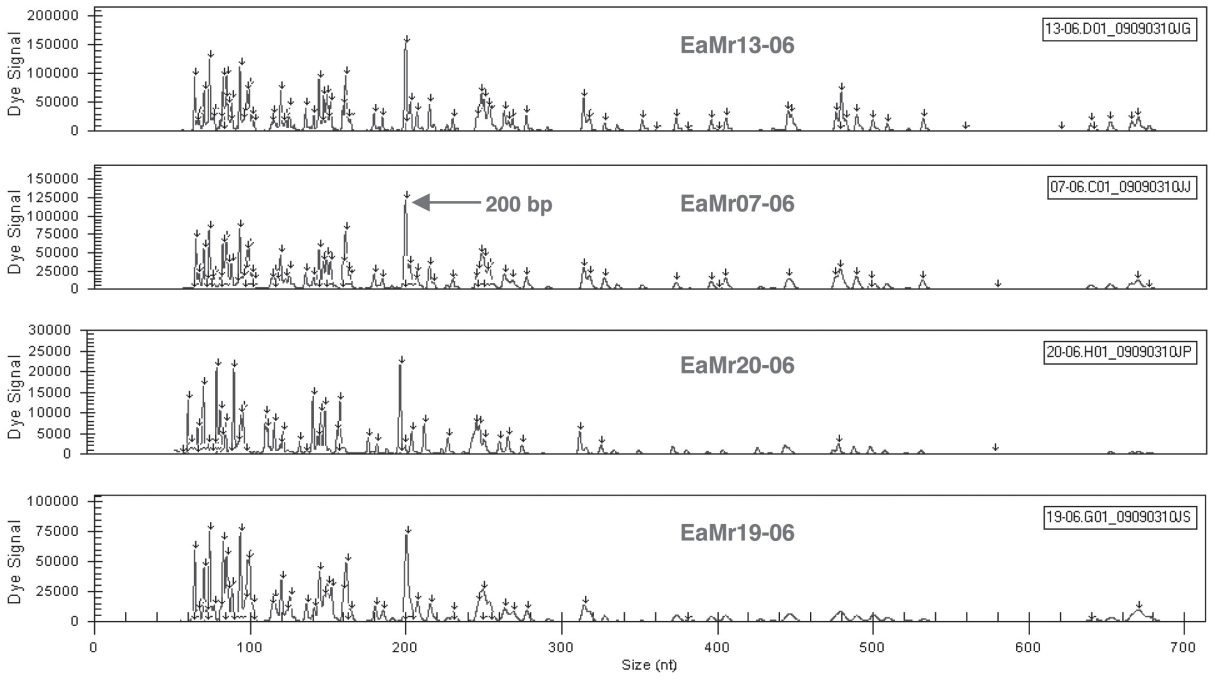

Figure 5. Electropherograms fAFLP after amplification using *Eco*RI+A plus *Mse*I+C primers.

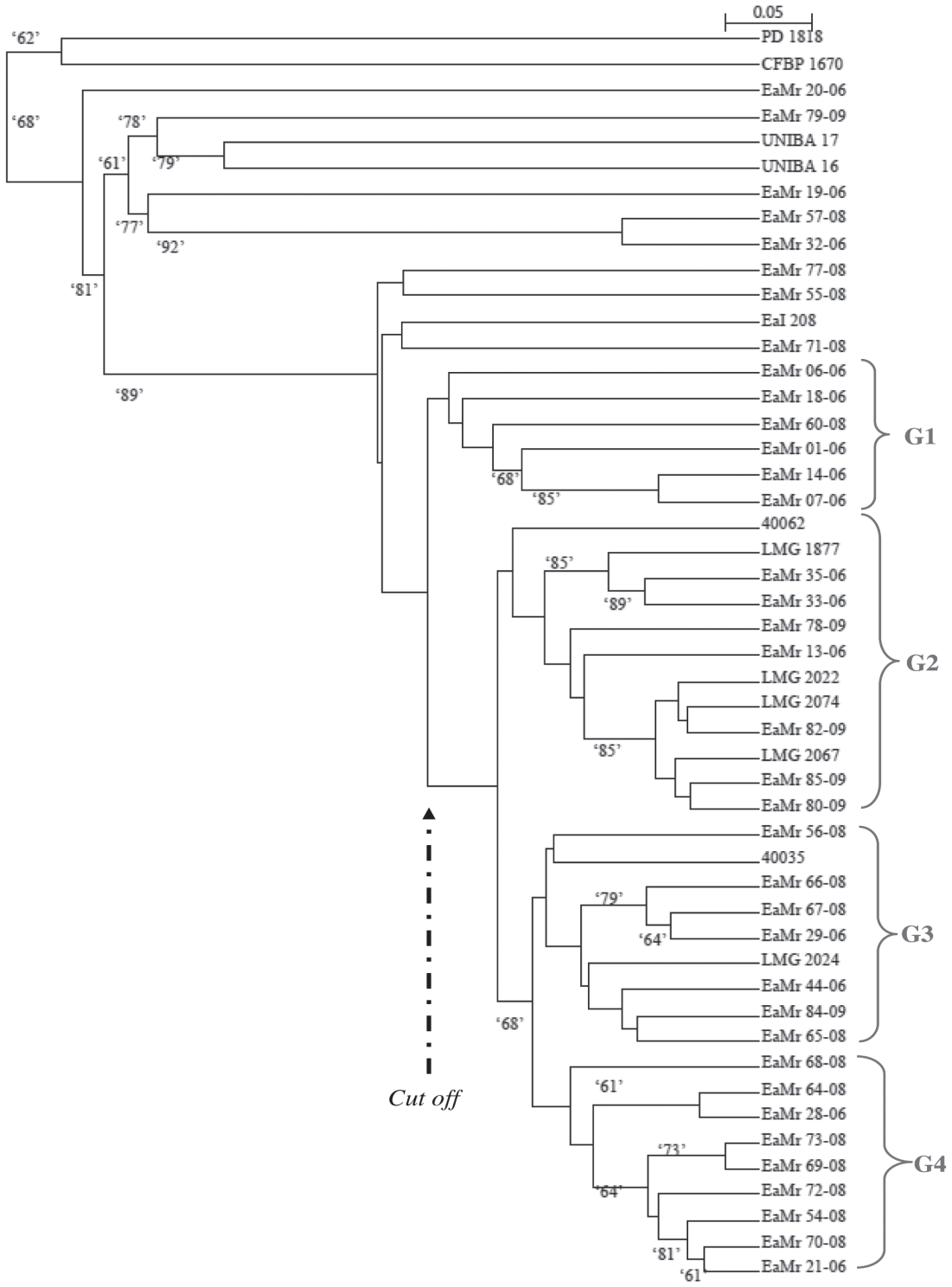

Figure 6. Dendrogram of *Erwinia amylovora* strains derived from fAFLP analysis calculated with unweighted pair-group method with average linkages (UPGMA) using the Jaccard's coefficient. Clusters (18.4% cut off) are indicated with letters: G1, G2, G3, G4, and the robustness of the tree was assessed by bootstrap analysis.

dustry. This includes an eradication of inoculum reservoirs by national phytosanitary authorities, promoting of quarantine measures to prevent introduction of the pathogen from areas where *E. amylovora* is present, and development of effective diagnostic protocols for detecting the bacteria even in samples with no visible symptoms. At the scientific research level, some investigations could strongly help the country to establish this long-term control. For instance, the assessment of genetic variability of the pathogen may provide the first step in understanding of the origin of the initial source of inoculum in Morocco and its dispersion routes.

By biochemical, pathogenicity and rep-PCR tests no characteristics have been found that can clearly distinguish strains by geographical origin, host or year of isolation.

The ability of AFLP to detect polymorphisms was compared to rep-PCR, which is simpler and faster for the analysis of large numbers of strains, although it is less discriminative.

As reported by Momol and Aldwinckle (2000) and by Rico *et al*. (2008), rep-PCR fingerprinting has proved to be of very limited value in establishing genealogies or phylogenetic relationships.

fAFLP analysis was used for the first time as a molecular approach to assess the variability of *E. amylovora* strains isolated in Morocco from different hosts and in different years. In this study, it was found that a dual restriction digest with *Eco*RI and *Mse*I as a common primer combination was an excellent approach to generate information-rich fAFLP patterns from all *E. amylovora* strains. The 48 Moroccan strains showed distinctive fAFLP patterns, typically ranging between 43 and 82 fragments in the selected size range of 60 to 700 bp. The fAFLP technique allowed the detection of an unprecedented number of distinctive bands/peaks in *E. amylovora* and proved to be a sensitive, rapid and useful tool for discriminating among strains of this pathogen. These results suggest that the single primer combination *Eco*RI+A and *Mse*I+C was sufficient to characterize and type bacteria to the subspecies level. By using the same subset of strains, it was found that fAFLP discriminatory power was higher than rep-PCR. In fact, fAFLP differentiated *E. amylovora* strains and grouped some of them into separate clades, just in one case (G1) partially corresponding to the host and country of origin. According to Cirvilleri *et al*. (2008), strain-specific discriminative peaks in strains isolated from the same host could be useful for strain identification and for epidemiological studies. On the other hand, the genetic characterization of *E. amylovora* by fAFLP indicated high variability among the strains tested that is coherent with the occurrence of different populations subjected to varying environmental selection. Even using a high resolution method, such as fAFLP, no obvious relation was detected between the strains grouped in each cluster and the host, place or year of isolation. However, the lack of correspondence between genealogy and geographical origin is not surprising and agrees with the results obtained using biochemical, physiological, serological or genetic characteristics (Brennan *et al*., 2002).

The results obtained in this study strongly confirm the usefulness of the fAFLP technique to differentiate among some *E. amylovora* strains, undistinguishable by rep-PCR, and suggest the occurrence of multiple introduction of the pathogen in Morocco. According to Rico *et al*. (2004), a significantly larger number of strains from different locations would be necessary to confirm the hypothesis of multiple introductions.

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Literature cited

Atanasova I., P. Kabadjova-Hristova, K. Stefanova, N. Bogatzevska and P. Moncheva, 2009. Differentiation of *Erwinia amylovora* strains from Bulgaria by PCR-RFLP analysis. *European Journal of Plant Pathology* 124, 451–456.

Beer S.V., J.H. Kim, H.L. Gustafson, C.H. Zumoff, R.J. Laby, A.J. Bogdanove, A. Tanii, O. Tamura, M.T. Mo-

mol and H.S. Aldwinckle, 1996. Characterization of bacteria that cause "bacterial shoot blight" in Japan. *Acta Horticulturae* 411, 179–181.

Bereswill S., A. Pahl, P. Bellemann, W. Zeller and K. Geider, 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. *Applied and Environmental Microbiology* 58, 3522–3526.

Bonn W.G. and T. Van der Zwet, 2000. Distribution and economic importance of fire blight. In: *Fire blight: the Disease and its Causative Agent Erwinia amylovora*. (Vanneste J.L., ed.). CABI publishing, Wallingford, UK, 37–53.

Brennan J.M., F.M. Doohan, D. Egan, H. Scanlan and D. Hayes, 2002. Characterization and differentiation of Irish *Erwinia amylovora* isolates. *Journal of Phytopathology* 150, 414–422.

Cirvilleri G., G. Scuderi, A. Bonaccorsi and M. Scortichini, 2006. Molecular characterization *of Pseudomonas syringae* pv. *syringae* strains from different host plants using fluorescent amplified fragment length polymorphism. *Journal of Plant Pathology* 88, 327–331.

Cirvilleri G., G. Scuderi, A. Bonaccorsi and M. Scortichini, 2007. Occurrence of *Pseudomonas syringae* pv. *coryli* on hazelnut orchards in sicily, Italy and characterization by fluorescent amplified fragment length polymorphism. *Journal of Phytopathology* 155, 397–402.

Cirvilleri G., G. Scuderi, A. Bonaccorsi and M. Scortichini, 2008. Analysis of *Pseudomonas syringae* populations and identification of strains as potential biocontrol agents against postharvest rot of different fruits. In: "Pseudomonas syringae *Pathovars and Related Pathogens-Identification, Epidemiology and Genomics*", (Fatmi M'B., Collmer A. Iacobellis N.S., Mansfield J.W., Murillo J., Schaad N.W., Ullrich M., ed.) Springer, Berlin, Germany, 125–144.

Clerc A., C. Manceau and X. Nesme, 1998. Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology* 64, 1180–1187.

Donat V., E.G. Biosca, J. Penalver and M.M. Lopez, 2007. Exploring diversity among Spanish strains of *Erwinia amylovora* and possible infection sources. *Journal of Applied Microbiology* 103, 1639–1649.

EPPO, 2004. EPPO standards: Diagnostic protocols for regulated pests. *EPPO Bulletin* 34, 155–157.

Fatmi M., 2009. Le feu bactérien au Maroc: Situation actuelle et stratégie de lutte. *Packinfo* N° 77 Avril 2009.

Fatmi M., M. Bougsiba and H. Saoud, 2008. First report of fire blight caused by *Erwinia amylovora* on pear, apple and quince in Morocco. *Plant Disease* 92, 314.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.

Holt J.G., 1994. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Williams and Wilkins, Baltimore, MA, USA, pp. 179.

Ishimaru C. and E. J. Klos, 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* 74, 1342–1345.

Janssen P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau and K. Kersters, 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142, 1881–1893.

Jock S., V. Donat, M.M. Lopez, C. Bazzi and K. Geider, 2002. Following spread of fire blight in western, central and southern Europe by molecular differentiation of *Erwinia amylov*ora strains with PFGE analysis. *Environmental Microbiology* 4, 106–114.

Jones A. L. and K. Geider, 2001. *Erwinia amylovora* group. In: *Laboratory guide for identification of plant pathogenic bacteria*. 3rd ed. (Schad N. W., Jones J.B. and Chun W., ed.). APS press, St. Paul, MN, USA, 40–54.

Kaneshiro W.S., M. Burger, B. G.Vine, A.S. de Silva and A.M. Alvarez, 2008. Characterization of *Erwinia chrysanthemi* from a bacterial heart rot of pineapple outbreak in Hawaii. *Plant Disease* 92, 1444–1450.

Kassama Y., P.J Rooney and R. Goodacre, 2002. Fluorescent amplified fragment length polymorphism probalistic database for identification of bacterial isolates from urinary tracts infections. *Journal of Clinical Microbiology* 40, 2795–2800.

Kim W.S., S. Jock, J.P. Paulin, S.L. Rhim and K. Geider, 2001. Molecular detection of differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Disease* 85, 1183–1188.

King E.O., M.K. Ward and D.E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* 44, 301–307.

Lehman S.M., W.S. Kim, A J. Castle and M. Svircev, 2008. Duplex real-time polymerase chain reaction reveals competition between *Erwinia amylovora* and *E. pyrifoliae* on pear blossoms. *Phytopathology* 98, 673–679.

Llop P., P. Caruso, J. Cubero, C. Morente and M.M. Lopez, 1999. A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods* 37, 23–31.

Llop P., V. Donat, M. Rodriguez, J. Cabrefiga, L. Ruz, J. Palomo, E. Montesinos and M. Lopez, 2006. An indigenous virulent strain of *Erwinia amylovora* lacking the 508 ubiquitous plasmid pEA29. *Phytopathology* 96, 900–907.

Maes M. and C. Crepel, 1997. Differential detection of *Erwinia amylovora* isolates from fruit trees and *Rubus* spp. based on ribosomal DNA and PCR. In: *Diagnosis and Identification of Plant Pathogens*. (H.W. Dehne H.W., Adam G., Diekmann M., Frahm J., Mauler–Machnik A., Van Halteren P., ed.). Kluwer Academic Publishers, Bonn, Germany, 437–439.

Maes M., P. Garbeva and C. Crepel, 1996. Identification and sensitive endophytic detection of the fire blight pathogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. *Plant Pathology* 45, 1139–1149.

Momol M.T and H.S. Aldwinckle, 2000. Genetic diversity

and host range of *Erwinia amylovora*. In: *Fire blight: the disease and its causative agent,* Erwinia amylovora. (Vanneste J.L., ed.). CABI publishing, Wallingford, UK, 55–72.

Momol M.T., E.A. Momol, W.F. Lamboy, J.L. Norelli, S.V. Beer and H.S. Aldwinckle, 1997. Characterization of *Erwinia amylovora* strains using random amplified polymorphic DNA fragments (RAPDs). *Journal of Applied Microbiology* 82, 389–398.

Momol M.T., J.L. Norelli and H.S. Aldwinckle, 1999. Evaluation of biological control agents, systemic acquired resistance inducers and bactericides for the control of fire blight on apple blossom. *Acta Horticulturae* 489, 591–595.

Ordax M., E. Marco–Noales, M.M. Lopez and E.G. Biosca, 2006. Copper induces a viable but nonculturable (VBNC) state in *Erwinia amylovora*. *Acta Horticulturae* 704, 205–210.

Rademaker J.L.W., B. Hoste, F.J. Louws, K. Kersters, J. Swings, L. Vauterin, P. Vauterin and F.J. de Bruijn, 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* 50, 665–677.

Restrepo S., M. Duque, J. Tohme, V. Verdier, 1999. AFLP fingerprinting: an efficient technique for detecting genetic variation of *Xanthomonas axonopodis* pv. *Manihotis*. *Microbiology* 145, 107–114.

Rhim S.L., B. Volksch, L. Gardan, J.P. Paulin, C. Langlotz, W.S. Kim and K. Geider, 1999. *Erwinia pyrifoliae*, an *Erwinia* species different from *Erwinia amylovora*, causes a necrotic disease of asian pear trees. *Plant Pathology* 48, 514–520.

Rico A., A. Ortiz-Barredo, E. Ritter and J. Murillo, 2004. Genetic characterization of *Erwinia amylovora* strains by amplified fragment length polymorphism (AFLP). *Journal of Applied Microbiology* 96, 302–310.

Rico A., M.E. Führer, A. Ortiz-Barredo and J. Murillo, 2008. Polymerase chain reaction fingerprinting of *Erwinia amylovora* has a limited phylogenetic value but allows the design of highly specific molecular markers. *Phytopathology* 98 (3), 260–269.

Roselló M., R. Christen, P. Llop, S. Ferrer, L. Garden and M.M. López, 2008. Description of *Erwinia piriflorinigrans* sp. nov., causal agent of pear blossom necrosis. *Acta Horticulturae* 793, 137–140.

Scuderi G., A. Bonaccorsi, S. Panebianco, A. Vitale, G. Polizzi and G. Cirvilleri, 2009. Some strains of *Burkholderia gladioli* are potential candidates for postharvest biocontrol of fungal rots in citrus and apple fruits. *Journal of Plant Pathology* 91, 207–213.

Smith H. and R. Lattimore, 1997. The search for rules for non tariff barriers: fire blight of apples. *International Agricultural Trade Research Consortium (IATRC) Meeting*, San Diego, CA, USA, December 14–16, 1997 [on line]. [http://researcharchive.lincoln.ac.nz/dspace/bitstream/10182/872/1/cd_dp_44 .pdf]

Van de Bilt J.L.J., J.H.J Derks, and J.D. Janse, 2008. Automated, high-throughput immunofluorescence staining: a new approach. *European Journal of Plant Pathology* 120, 91–96.

Van der Zwet T., 2002. Present worldwide distribution of fire blight. *Acta Horticulturae (ISHS)* 590, 33–34.

Van der Zwet T., 2006. Present worldwide distribution of fire blight and closely related diseases. *Acta Horticulturae (ISHS)* 704, 35–36.

Van der Zwet T. and J.M. Wells, 1993. Application of fatty acid class analyses for the detection and identification of *Erwinia amylovora*. *Acta Horticulturae* 338, 233.

Vanneste J.L., 2000. What is fire blight? Who is *Erwinia amylovora*? How to control it? In: *Fire blight: the disease and its causative agent Erwinia amylovora*. (Vanneste J.L., ed.). CABI publishing, Wallingford, UK, 1–6.

Vantomme R., J. Swings, M. Goor, K. Kersters and J. De Ley, 1982. Phytopathological, Serological, Biochemical and protein electrophoretic characterization of *Erwinia amylovora* strains isolated in Belgium. *Phytopathologische Zeitschrift* 103, 349–360.

Vantomme R., C. Rijckaert, J. Swings and J. De Ley, 1986. Characterization of further *Erwinia amylovora* strains and the application of the API 20E System in Diagnosis. *Journal of Phytopathology* 117, 34–42.

Versalovic J., T. Koeuth and J.R. Lupski, 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* 19, 6823–6831.

Winslow C.E.A., J. Broadhurst, R.E. Buchanan., C. Krumwiede, L.A. Rogers and G.H. Smith, 1920. The families and genera of the bacteria. Final report of the Committee of the Society of American Bacteriologists on characterization and classification of bacterial types. *Journal of Bacteriology* 5, 191–229.

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