

GENETIC VARIABILITY OF *XANTHOMONAS FRAGARIAE* STRAINS OBTAINED FROM FIELD OUTBREAKS AND CULTURE COLLECTIONS AS REVEALED BY REPETITIVE-SEQUENCE PCR AND AFLP

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SUMMARY

The genetic variability of 43 *Xanthomonas fragariae* strains isolated in Italy during three different field outbreaks was compared with 20 representative strains previously isolated in Australia, USA, Brazil, Greece, New Zealand and Italy, and obtained from international culture collections. Typing was performed by repetitive-sequence PCR (rep-PCR) using BOX, ERIC and REP primer sets and amplified fragment length polymorphism (AFLP) analysis. Both techniques revealed that *X. fragariae* strains can be divided into different subgroups. No correlation between DNA amplification product patterns and geographic areas of origin was found. AFLP analysis revealed the existence of two major groups of strains related at a level of 73%. BOX, ERIC and rep-PCR assays identified five, three and two genotypes, respectively. The grouping of strains inferred by these two techniques was not mutually consistent. The strains of the three outbreaks in Italy showed in general a very similar banding pattern. No host specificity groups could be identified since two *X. fragariae* strains isolated from *Fragaria vesca* and one strain obtained from *Fragaria chiloensis* showed a similar banding pattern, like other strains isolated from *Fragaria x ananassa*. As determined by pathogenicity tests, no difference in virulence could be observed among strains of the classified groups. For the improvement of *X. fragariae* detection in strawberry propagative material, the relevant genetic variability of the pathogen should be taken into consideration.

Key words: Phytobacteria, genotypes, identification, genetic variability, rep-PCR, AFLP analysis.

INTRODUCTION

Xanthomonas fragariae Kennedy *et* King, the causal agent of angular leaf spot, is a pathogen spread in all major areas of strawberry (*Fragaria x ananassa* Duch.) cultivation. In the countries belonging to the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (EU), *X. fragariae* has A2 quarantine status (Smith *et al.*, 1992). The isolation of this bacterial species is not always easy, even from symptomatic plant material, and its detection by serological techniques may yield doubtful results (Stöger and Ruppitsch, 2004). Knowledge of the genetic variability of a pathogen is a fundamental pre-requisite to develop reliable diagnostic techniques. Variability within species has been determined by means of several phenotypic and genetic techniques such as fatty acid analysis (Roberts *et al.*, 1998; Janse *et al.*, 2001), whole-cell protein profiling (Janse *et al.*, 2001), random amplified polymorphic DNA (Pooler *et al.*, 1996), restriction fragment length polymorphism (Roberts *et al.*, 1998), repetitive-sequence PCR (rep-PCR) (Opgenorth *et al.*, 1996; Pooler *et al.*, 1996; Gillings *et al.*, 1998). All techniques revealed a certain degree of variability and no clear-cut relationships was found between the geographic origin of *X. fragariae* strains and the diverse clusters obtained in the analysis.

However, all studies have dealt with a limited number of strains collected in different years. In addition, the genetic variability of strains isolated in the same area during different outbreaks was assessed only once using rep-PCR (Opgenorth *et al.*, 1996). On the other hand, genetic variability of xanthomonads isolated from the same field has been observed, representing a first step for studying the population structure of a pathogen (Scortichini *et al.*, 2001).

In this study, we have assessed the genetic variability of 63 strains of *X. fragariae* obtained from three field outbreaks of angular leaf spot occurred in Italy and of strains from international culture collections, using rep-PCR with BOX, ERIC and REP primer sets and amplified fragment length polymorphism (AFLP) analysis. The first technique has previously been employed to investigate *X. fragariae* variability (Opgenorth *et al.*, 1996;

Pooler *et al.*, 1996; Gillings *et al.*, 1998), whereas AFLP, a powerful technique enabling the characterization of bacterial pathogens at the strain level, has been used to infer taxonomic relationships between *X. fragariae* and *X. arboricola* pv. *fragariae* (Janse *et al.*, 2001), two xanthomonads pathogenic to strawberry.

MATERIALS AND METHODS

Recovery and identification of *X. fragariae* isolates.

The 63 *X. fragariae* strains used in this study are listed in Table 1. Strains not coming from culture collections were isolated in northern Italy (i.e. Emilia-Romagna region) during three field outbreaks in 2003, 2005 and 2006, as described by Lopez *et al.* (2006). Wilbrink medium was used for the isolation from fresh, water-soaked angular lesions and identification was achieved by DAS-ELISA using a commercial kit (Bioreba, Switzerland), immunofluorescent antibody staining (IFAS) with a polyclonal antiserum raised against *X. fragariae* NCPPB 2473, isolated in Italy in 1972, and by multiplex-PCR using the three sets of primers suggested by Pooler *et al.* (1996).

With some representative strains belonging to the different groups determined by rep-PCR and AFLP analysis, pathogenicity tests on strawberry plants cv. Pajaro were made, as reported by Pooler *et al.* (1996).

REP-PCR and AFLP analysis. rep-PCR and AFLP analysis were carried out on the 63 *X. fragariae* strains. In particular, rep-PCR was performed using BOX, ERIC and REP primer sets as described elsewhere (Scortichini *et al.*, 2001; Scortichini and Rossi, 2003), whereas a AFLP microorganism kit (Invitrogen, USA) was used for AFLP analysis (Vos *et al.*, 1995) following manufacturer's instructions. The *EcoRI* primers supplied with the kit were replaced by IRD-800 labelled *EcoRI* primers for analysis on an automated DNA sequencer. Among several AFLP primer combinations tested, the set E-O/M-G was finally chosen for comparing the bacterial strains, due to their higher discriminating resolution. Analysis of AFLP gels and transformation of the AFLP band pattern into a binary code was done with the aid of the AFLP analysis software SAGA-MX (LI-COR Bioscience, USA). For AFLP analysis, a dendrogram based on UPGMA analysis using the Dice's coefficient was built using a NTSYS software (Exeter, USA), version 2.01.

RESULTS

***X. fragariae* isolation and identification.** After five days incubation at 27°C, light-yellow bacterial colonies 1 mm in diameter developed on Wilbrink medium. Single colonies were streaked in purity on the same medium

for serological and molecular assays. Only the isolates positive to DAS-ELISA and IFAS, and showing the three expected bands of 300, 550 and 615 bp upon multiplex-PCR, were retained as *X. fragariae*. A total of 43 strains were collected. Ten strains covering the range of genetic variability as determined by rep-PCR and AFLP were chosen for pathogenicity tests. All strains caused the same kind of symptoms on inoculated strawberry leaves (i.e. angular, water-soaked spots with subsequent necrosis) three weeks after inoculation. No differential virulence among the tested strains was noticed.

REP-PCR and AFLP analysis. Rep-PCR yielded reproducible results in typing *X. fragariae* strains. BOX-PCR, ERIC-PCR and rep-PCR revealed five, three and two genotypes, respectively. Variability in the banding pattern was observed between 200 and 2,000 bp, indicating that BOX-PCR was more discriminating than ERIC-PCR and rep-PCR as it identified more groups of strains. A representative BOX-PCR gel is shown in Fig. 1. No correlation between grouping and geographic origin of the strains was observed. However, groups A and B contained only strains isolated in Italy from field outbreaks.

UPGMA analysis performed with Dice's coefficient following AFLP, revealed two major groups of *X. fragariae* strains, denoted A and B, with a 73% genetic similarity. Each group, in turn, could be subdivided into two subgroups, i.e. A1, A2 and B1, B2 (Fig. 2). The strains from the three different field outbreaks recorded in Italy did not cluster into outbreak specific subgroups. AFLP cluster A2, that contained the majority of the isolates from the international strain collection, did not comprise Italian isolates collected during the outbreaks

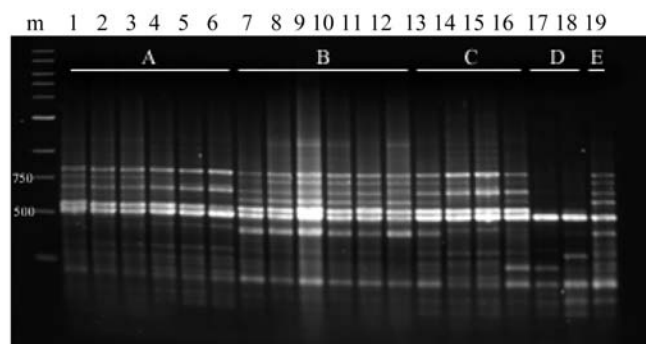


Fig. 1. Representative genetic fingerprinting of *Xanthomonas fragariae* strains obtained with BOX-PCR showing the presence of five genotypes. Lane m: molecular marker 1 kb ladder (Promega, USA). Group A. Lane 1, Xf 9; lane 2, Xf 10; lane 3, Xf 15; lane 4, Xf 16; lane 5, Xf 17; lane 6, Xf 27. Group B. Lane 7, Xf 11; lane 8, Xf 12; lane 9, Xf 13; lane 10, Xf 14; lane 11, Xf 19; lane 12, Xf 23. Group C. Lane 13, Xf 27; lane 14, Xf 29; lane 15, Xf 37; lane 16, NCPPB 2949. Group D. Lane 17, DAR 26919; lane 18, DAR 69862. Group E. Lane 19, DAR 69815. Pathogenicity tests were made with isolates Xf9, Xf10, Xf11, Xf27, DAR 26919 and DAR 69862.

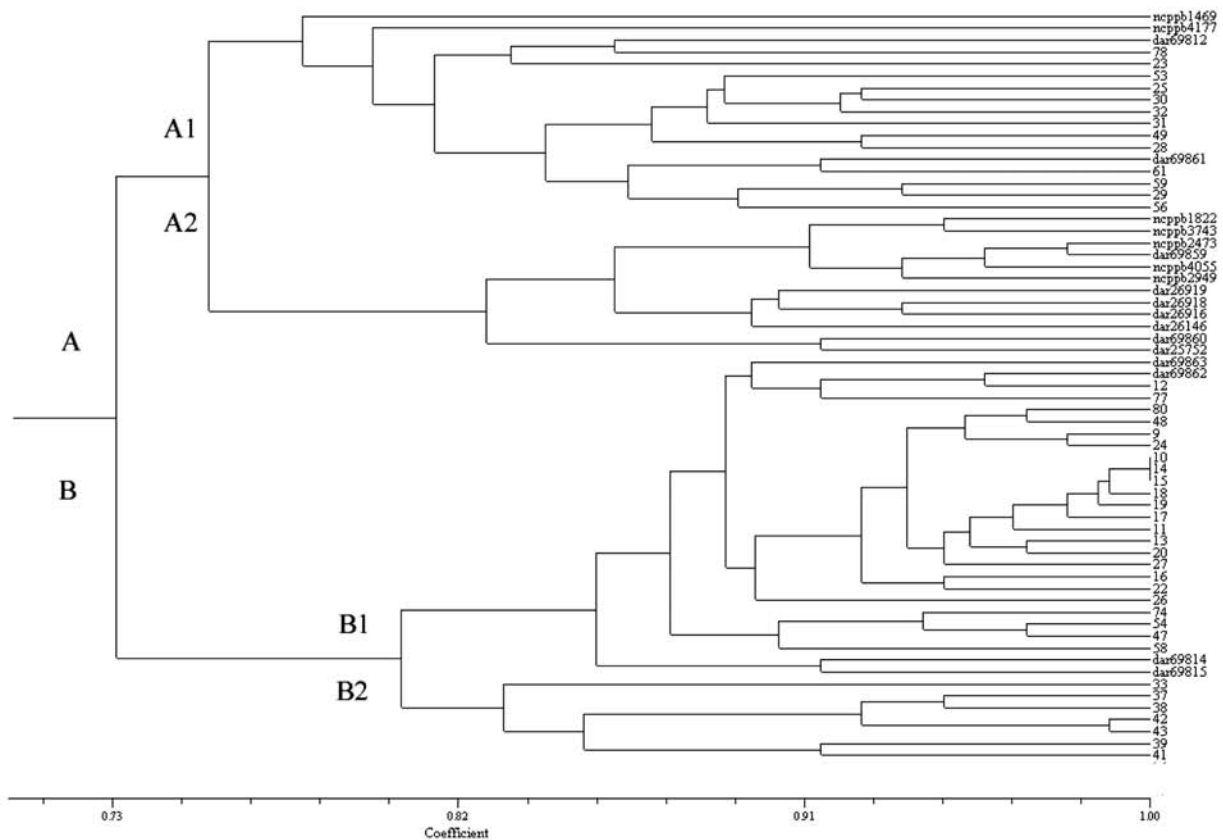


Fig. 2. Dendrogram of genetic relationships among *Xanthomonas fragariae* strains, obtained upon AFLP analysis and unweighted pair-group method with average linkage (UPGMA) using Dice's coefficient.

of 2003, 2005, and 2006. In fact, 66.7% of the strains from the 2003 and 2005 outbreaks belonged to AFLP cluster B1, and 33.4% to AFLP cluster A1. As to the 2006 outbreak, seven strains (28%) belonged to AFLP cluster B2, seven strains (28%) to AFLP cluster A1, and 11 strains (44%) to AFLP cluster B1.

With the exception of the AFLP cluster B2, no member of which was present in the international strain collection, no correlation between strains of the international collection and their geographic origin was found. The AFLP cluster B2 contained the two *X. fragariae* strains isolated from *F. vesca*, one strain from *F. chiloensis* and three other *X. fragariae* strains recovered from *Fragaria x ananassa*.

Strain typing obtained with rep-PCR and AFLP analysis was not mutually consistent. However, by combining the results obtained with these techniques (i.e. clustering of the strains), each *X. fragariae* strain could be genetically typed (Table 1).

DISCUSSION

This study has confirmed that genetic variability exists within *X. fragariae* populations. However, as previ-

ously shown by DNA-DNA reassociation (Roberts *et al.*, 1998), no clear-cut indication of the presence of distinct species or pathovars was found, since AFLP analysis showed an overall genetic similarity of 73%.

As observed with other techniques (i.e. fatty acid analysis, RFLP, rep-PCR) (Pooler *et al.*, 1996; Roberts *et al.*, 1998; this study), AFLP revealed that *X. fragariae* can be grouped into different clusters, with no evident relationships between AFLP groups and the geographic origin of the strains. However, whereas the majority of the international strains investigated in this study belonged to AFLP cluster A2, none of the Italian outbreak isolates was present in the same group. In fact, the majority of the Italian isolates clustered in AFLP B1, which comprised also four international strains (two each from Australia and USA). However, the latter strains could be differentiated from the Italian ones by a distinct BOX-PCR pattern.

Restriction analysis of genomic DNA by pulsed field gel electrophoresis showed that the type-strain of the species, NCPPB 1469, deviates from the other *X. fragariae* strains (Roberts *et al.*, 1998). UPGMA analysis performed with AFLP data, confirmed the distinctiveness of this strain, which was the most divergent in the AFLP subgroup A1. Also BOX-PCR proved effective in differentiating *X. fragariae* strains in five genotypes.

Table 1. List of *Xanthomonas fragariae* strains used in this study and repetitive-sequence PCR (BOX, ERIC and REP primer sets) and AFLP grouping.

Strain	Origin	Year of isolation	BOX	ERIC	REP	AFLP
NCPPB 1469 ^T	USA	1962	D	B	A	A1
NCPPB 1822	USA	1966	D	B	A	A2
NCPPB 2473	Italy	1972	C	A	A	A2
NCPPB 2949	Australia	1977	C	A	A	A2
NCPPB 3743	Brazil	1962	D	A	A	A2
NCPPB 4055	Greece	1962	C	A	A	A2
NCPPB 4177	Unknown	1962	B	A	A	A1
DAR 25752	Australia	1975	D	C	A	A2
DAR 26146	Australia	1975	D	C	A	A2
DAR 26916	New Zealand	1971	D	C	A	A2
DAR 26918	Australia	1975	D	C	A	A2
DAR 26919	Australia	1975	D	C	A	A2
DAR 69812	Australia	1994	E	A	A	A1
DAR 69814	Australia	1994	E	A	A	B1
DAR 69815	Australia	1994	E	A	A	B1
DAR 69859	Brazil	1977	D	C	A	A2
DAR 69860	Brazil	1977	D	C	A	A2
DAR 69861	USA	unknown	D	C	A	A1
DAR 69862	USA	unknown	D	C	A	B1
DAR 69863	USA	unknown	D	C	A	B1
Xf 9	Italy	2005	A	A	A	B1
Xf 10	Italy	2005	A	A	A	B1
Xf 11	Italy	2005	B	A	A	B1
Xf 12	Italy	2005	B	A	A	B1
Xf 13	Italy	2005	B	A	A	B1
Xf 14	Italy	2006	B	A	A	B1
Xf 15	Italy	2006	A	A	A	B1
Xf 16	Italy	2006	A	A	A	B1
Xf 17	Italy	2006	A	A	A	B1
Xf 18	Italy	2006	B	A	B	B1
Xf 19	Italy	2006	B	A	A	B1
Xf 20	Italy	2006	A	A	A	B1
Xf 22	Italy	2006	A	A	A	B1
Xf 23	Italy	2006	B	A	A	A1
Xf 24	Italy	2006	A	A	A	B1
Xf 25	Italy	2006	A	A	A	A1
Xf 26	Italy	2006	A	B	A	B1
Xf 27	Italy	2006	C	A	A	B1
Xf 28	Italy	2006	A	A	A	A1
Xf 29	Italy	2006	C	A	A	A1
Xf 30	Italy	2006	A	A	A	A1
Xf 31	Italy	2006	A	A	A	A1
Xf 32	Italy	2006	A	A	A	A1
Xf 33	Italy	2006	A	A	A	B2
Xf 37	Italy	2006	C	A	A	B2
Xf 38	Italy	2006	B	A	A	B2
Xf 39	Italy	2006	A	A	A	B2
Xf 41 *	Italy	2006	D	A	A	B2
Xf 42**	Italy	2006	D	A	A	B2
Xf 43**	Italy	2006	D	A	B	B2
Xf 47	Italy	2005	A	A	A	B1
Xf 48	Italy	2005	A	A	A	B1
Xf 49	Italy	2005	E	A	A	A1
Xf 53	Italy	2005	A	A	A	A1
Xf 54	Italy	2005	A	A	A	B1
Xf 56	Italy	2005	A	A	A	A1
Xf 58	Italy	2005	A	A	A	B1
Xf 59	Italy	2005	A	A	A	A1
Xf 61	Italy	2005	A	A	A	A1
Xf 74	Italy	2005	A	A	A	B1
Xf 77	Italy	2003	A	A	A	B1
Xf 78	Italy	2003	A	A	A	A1
Xf 80	Italy	2003	A	A	A	B1

^T: type-strain; *: isolated from *Fragaria chiloensis*; **: isolated from *Fragaria vesca*; NCPPB: National Collection of Plant Pathogenic Bacteria, CSL, York, UK; DAR: Australian Collection of Plant Pathogenic Bacteria, Rydalmere, Australia

Rep-PCR using ERIC and REP primer sets has been used for the rapid identification of *X. fragariae* field isolates (Opgenorth *et al.*, 1996). Our study indicates that BOX-PCR is even more efficient in identifying similarities and differences among the strains.

Using BOX-PCR and ERIC-PCR, Gillings *et al.* (1998) differentiated two diverse outbreaks of strawberry angular leaf spot in Australia. In our study, both BOX-PCR and AFLP analysis confirmed the distinctiveness of some strains from these two outbreaks. A certain degree of differentiation was observed for the *X. fragariae* strains isolated from three field outbreaks in Italy. This may indicate a higher genetic variability of the infective strains found in consecutive years in Italy than that observed in other epidemics in California (Opgenorth *et al.*, 1996) and in Australia (Gillings *et al.*, 1989).

X. fragariae strains isolated from *F. vesca* and *F. chiloensis* grouped into their own AFLP cluster, together with a few other strains obtained from *Fragaria x ananassa*. However, more accurate studies with a larger number of strains are needed for clearly defining the genetic variability of *X. fragariae* strains isolated from wild *Fragaria* spp.

Finally, the relevant genetic variability existing within *X. fragariae* populations should be taken into account to improve the effectiveness of detection. In fact, routine detection of angular leaf spot in strawberry propagative material is still mainly based on the use of polyclonal antisera that are currently raised with antigens from single bacterial strains. False negative reactions in ELISA and immunofluorescence tests can therefore occur if *X. fragariae* strains with features differing from those of the strain used for immunization are present in the analyzed sample. The situation is even more stringent in the case of PCR-based detection techniques.

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