MOLECULAR CHARACTERIZATION OF TUNISIAN STRAINS OF *ERWINIA AMYLOVORA*

S. Dardouri[1,2,*], S. Chehimi[1,*], J. Murillo[3] and M.R. Hajlaoui[1]

[1]Laboratoire de Biotechnologie Appliquée à l'Agriculture, INRA Tunisie, 1004 Hedi Karray, Tunis, Tunisia
[2]Faculté des Sciences Mathématiques, Physiques et Naturelles de Tunis, Campus Universitaire 2092 El Manar, Tunis, Tunisia
[3]Laboratorio de Patología Vegetal, Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Spain
(*) These authors contributed equally to this work.

SUMMARY

The present study focused on the molecular characterization of a collection of *Erwinia amylovora* isolates recovered from different outbreaks in Tunisia between 2012 and 2014. Analysis of 54 isolates, including the reference type strain CFBP 1430, revealed that all Tunisian isolates produced the expected amplicons with diverse primer pairs routinely used for molecular diagnostics of *E. amylovora.* We also evaluated the genetic variability of these isolates by PCR fingerprinting, using specific primers for clustered regularly interspaced short palindromic repeats (CRISPRs) and for variable number of tandem repeats (VNTR) sequences. For the first method, our results revealed that all the primers used, except those for CRISPR3, which produced an identical amplicon for all isolates, showed some variability among Tunisian isolates. For the second method, forty-nine isolates showed the same fingerprint patterns as the reference type strain CFBP 1430 with all the primers used, whereas four of the isolates showed very divergent patterns. These results suggest that there has been a main introduction of European-type isolates in Tunisia, and possibly a few mutations or other independent introductions of the pathogen. Additionally, these results indicate that PCR fingerprinting using VNTR markers is a most useful tool for discriminating among *E. amylovora* strains and for their identification in epidemiological studies.

Keywords: fire blight, *Erwinia amylovora*, PCR, CRISPR, VNTR primers, fingerprinting, epidemiology.

Corresponding author: S. Dardouri
Fax: + 216 71 235 317
E-mail: sanadardouri@gmail.com

INTRODUCTION

Erwinia amylovora (Burill) Winslow *et al.* is the causal agent of the destructive fire blight disease that affects apple, pear and other plants in the family Rosaceae. Fire blight is native to North America but it has spread to many parts of the world, including Europe and many Mediterranean countries (Bonn and Vander Zwet, 2000). In Tunisia, it was first reported in spring 2012 in pear trees (*Pyrus communis* cv. Alexandrine, Williams) (Rhouma *et al.,* 2014). Fire blight has already caused large economic losses in apple and pear in Tunisia. One of the main problems hampering the efficient application of appropriate prevention is the initially unnoticed entry of this disease into a new country; therefore, its early spread and introduction often remain a matter for speculation. Knowledge on the epidemiology and genetic characterization of individual outbreaks may answer open questions on how bacteria and bacterial diseases are spread on a global scale or are dispersed naturally at the local level.

Techniques used to evaluate genetic diversity are important in epidemiological studies of *E. amylovora* and can be used to detect and identify possible sources of primary infection as well as in mapping bacterial genes (Bowditch *et al*., 1993; Hannou *et al*., 2013). Additionally, they could provide helpful information to differentiate strains and to evaluate their phylogenetic relationships. Based on previous molecular techniques such as PCR-ribotyping (Vanneste, 1995; Momol *et al.,* 1999; Donat *et al.,* 2007; Powney *et al.,* 2011), pulsed field gel electrophoresis (PFGE) (Jock *et al.,* 2002; Jock and Geider, 2004), rep-PCR (Momol *et al*., 1997; Barionovi *et al*., 2006; Rico *et al*., 2008), random amplified polymorphic DNA fragments (RAPDs), amplified rDNA restriction analysis (ARDRA) (Momol *et al.,* 2000), amplified fragment length polymorphism (AFLP) (Rico *et al.,* 2004; Donat *et al.,* 2007), and restriction fragment length polymorphism (RFLP) (Barionovi *et al.,* 2006), *E. amylovora* has been considered a fairly homogenous species. This limited genetic diversity makes therefore the characterization of the pathogen populations by traditional typing methods more difficult.

Nevertheless, methodologies developed on the basis of released pan-genome *E. amylovora* sequences such as clustered regularly interspaced short palindromic repeats

(CRISPRs) (Sorek *et al*., 2008; Rezzonico *et al*., 2011; McGhee and Sundin, 2012; Shariat and Dudley, 2014) and variable number of tandem repeats (VNTR) (Hannou *et al*., 2013; Bühlmann *et al*., 2014) offer new sources of variability for epidemiological studies. The number of CRISPR arrays in a bacterial chromosome is variable, and the content of each array can differ in both number of repeats and in the presence or absence of specific spacers. *E. amylovora* strains contain three CRISPR loci that are separated by regularly-sized non-repetitive spacer sequences (McGhee and Sundin, 2012; Pulwaska and Sobiczewski, 2012). Variable number of tandem repeats (VNTR) have been found in all organisms and recognized as highly polymorphic loci. They can vary on a large scale in their characteristics (unit size, number of repeats, repeat mode, and complexity) and can be very useful for typing bacteria (Onteniente *et al.,* 2003; Yazdankhah and Linstedt, 2007; Dreo *et al.,* 2011; Hannou *et al.,* 2013; Drenova *et al*., 2016).

The objectives of this study were: (i) the molecular characterization of 53 isolates from different outbreaks of fire blight in Tunisia, and (ii) exploring the genetic diversity within this collection by PCR fingerprinting using primers targeting CRISPR loci and VNTR sequences. This work constitutes the first genetic characterization of Tunisian *E. amylovora* strains.

MATERIALS AND METHODS

Sampling and isolation of *E. amylovora*. Details and characteristics of *E. amylovora* isolates used in this work are listed in Table 1; the type strain *E. amylovora* CFBP 1430 was used as a reference control for all analyses. A total of 53 *E. amylovora* isolates were obtained and purified using standard protocols (Jones and Geider, 2001) from fresh, typical lesions of fire blight (reddish to brown color of affected shoots) on pear and apple between 2012 and 2014 from different regions in Tunisia. For routine maintenance and examination of colony morphology, isolates were propagated for 24 to 48 h at 26°C on King's B, CCT (Ishimaru and Klos, 1984) and Sucrose Nutrient Agar (SNA) media according to the EPPO protocol (2013). All strains were stored in 15% glycerol at −80°C.

Hypersensitivity test was done on tobacco (*Nicotiana tabacum* cv. Samsun NN). Bacterial suspensions of 1×10^9 cfu/ml (OD at 620 nm = 1.0) were injected into the intracellular space of the adult leaves with a 25 GA 5/8 0.5 × 16 needle and a syringe. Complete collapse of the infiltrated tissue after 24 h at room temperature was recorded as positive. The pathogenicity of the strains was examined by inoculation of wounded immature pear fruits (cv. Williams) using 10 µl of 1×10^9 cfu/ml bacterial suspensions. Strain CFBP 1430 and water were used as controls. Pear fruits were incubated in a humid room at 25°C for 3-5 days (Anonymous, 2013).

Molecular identification. For the identification of isolates as *E. amylovora* by PCR, we used three specific sets of primers: AJ75/AJ76 that amplify a 844 bp fragment from plasmid pEA29 (McManus and Jones, 1995) and G1-F/G2-R and FER1-F/rgER2-R amplifying two chromosomal regions (187 bp and 458 bp, respectively) (Taylor *et al.,* 2001; Obradovic *et al.,* 2007).

To further confirm the identity of the 53 strains, two other primer sets were used. The first was used to amplify the housekeeping gene *rpoB* according to Rezzonico *et al*. (2012) with slight modification of the primers: ErwRpoB-F (5'-CGACAACAAGCTGCAGATGGA-3') and ErwRpoB-R (5'-CGTCGAGGTTGGTGTTTGCCT-3'). The second were the species-specific primers EAPSGL3961 and EAPSGL4610c that amplify a 650 bp fragment (Wensing *et al*., 2011). All PCR reactions were carried out in a total volume of 25 µl containing 10 pmoles of each primer, 2× BioMix (Bioline) and 2.5 µl of purified bacterial DNA. Sterile water was used as DNA-negative control in every PCR run. Amplifications were performed using a 1000 Touch (BioRaD) PCR Thermocycler with one cycle of 5 min at 94°C, followed by: 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min for AJ75/AJ76, ERWRPOB-F/R and EAPSGL3961/4610c; 40 cycles at 94°C for 3 min, 60°C for 30 s and 72°C for 1 min for G1-F/G2-R; 41 cycles at 94°C for 10 s, 60°C for 10 s and 72°C for 30 s for FER1-F/rgER2-R with a final extension step at 72°C for 5 min for all PCR reactions. Then, PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A 100 bp DNA ladder (Invitrogen) was used as a size marker. PCR amplification products of the selected isolates (2, 16, 21 and 34) were purified with an Extraction Mini Kit (5 Prime) according to the manufacturer's instructions and sequenced by Macrogen Europe (The Netherlands). Sequence similarity was determined using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned using the Multalin software (http://bioinfo.genopoletoulouse.prd.fr/multalin/multalin.html).

PCR fingerprinting. To explore the diversity of the Tunisian strains of *E. amylovora,* two different fingerprinting approaches (CRISPRs and VNTRs) were used.

For CRISPR loci, PCR fingerprinting was accomplished using 20 pmoles of each primer pair: CRISPR 1 (CR1-F1/C1-R0), CRISPR 2 (Cr2-F1/C2-R1) and CRISPR 3 (CR3-F1/CR3-R1), [2X] BioMix (Bioline) and 1.5 µl of purified DNA. Cycling conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 s, annealing temperatures of 58°C (CRISPR 1 and 2) or 55°C (CRISPR 3) for 30 s, followed by 72°C for 4 min (CRISPR 1 and 2) or 45 s (CRISPR 3) with a final extension time for 7 min at 72°C (McGhee and Sundin, 2012). Each PCR reaction was performed at least twice in separate experiments.

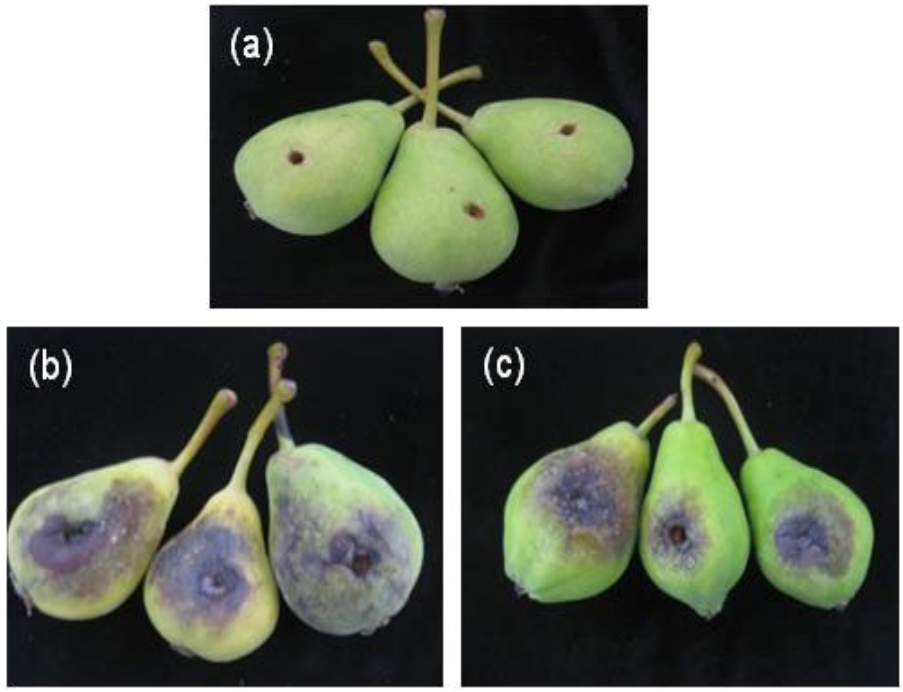

Fig. 1. Pathogenicity test carried out in immature pear fruits (Williams variety). (a) negative control; (b) inoculated with the reference type strain CFBP 1430 and (c) inoculated with strain N°45.

For VNTRs, PCR fingerprinting based on three VNTRs sequences (VNTR4, VNTR5 and VNTR6) was used according to Hannou *et al.* (2013). The PCR reaction was performed in a final volume of 50 µl containing 5 µl of purified bacterial DNA, 10 pmoles of a single VNTR primer and [2×] BioMix (Bioline). The cycling parameters were: an initial denaturation for 3 min at 94°C, 40 cycles of 94°C for 1 min, 45°C for 1 min, 65°C for 8 min and a final extension for 16 min at 65°C. Amplification products were separated by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator and photographed. A 1 kb DNA ladder (Invitrogen) was used as a size marker. PCR amplifications were repeated in duplicate.

RESULTS AND DISCUSSION

Isolation and identification of *E. amylovora* strains. In Tunisia, symptoms of fire blight were observed for the first time in spring 2012 in pear trees (Rhouma *et al.*, 2014). Based on a survey conducted in the main pear and apple growing areas in Tunisia from 2012 to 2014, samples were taken from different symptomatic trees. A collection of 53 isolates was obtained on King's B medium considering one isolate per tree. Inoculation of pure cultures of the isolated strains showed that all of them grew on CCT and SNA media. Additionally, all the tested strains produced a hypersensitive response in tobacco leaves 24 h after inoculation and induced different degrees of disease in the immature pear fruit assay (Fig. 1).

For PCR tests, all the used strains produced the expected amplicons (187 bp, 458 bp and 650 bp), which are specific for *E. amylovora* (Taylor *et al.*, 2001; Obradovic *et al.*, 2007; Wensing *et al.,* 2011). Similarly, the 844 bp fragment was successfully amplified for all the strains, thus confirming the presence of the plasmid pEA 29 in all the strains (McManus and Jones, 1995). Likewise, the 53 strains produced the expected 1.2 kb amplicon with the primer pair ERWrpoB-F/R developed in this work. Amplicon sequences from strains 2, 16, 21 and 34 (GenBank accession Nos. KU715987, KU715988, KU715989 and KU715990, respectively) showed 100% identity with strain CFBP 1430 and no more than 95% identity with strains of the phylogenetically closest species, *E. pyrifoliae*. Therefore, following international recommendations (Anonymous, 2013), the 53 isolates used in this work were identified as *E. amylovora* by biochemical, PCR and pathogenicity tests.

PCR patterns of CRISPR spacers. Genetic variability of 53 Tunisian isolates of *E. amylovora* from different fire blight outbreaks was assessed by PCR amplification of CRISPR loci. Results recorded with primers bordering the CRISPR 1 and 2 array sequences revealed the presence of a major homogeneous group of strains (49 strains) that produced amplicons of the same size as the reference strain CFBP 1430 and the existence of four different profiles (Table 1; Fig. 2). In fact, with CRISPR 1, isolates number 2, 16, 21 and 34 repetitively produced different amplification patterns, with weak or no amplicon (strains 2, 21 and 34) or two amplification bands (strain 16) (Fig. 2a). These results suggest a higher degree of variability than

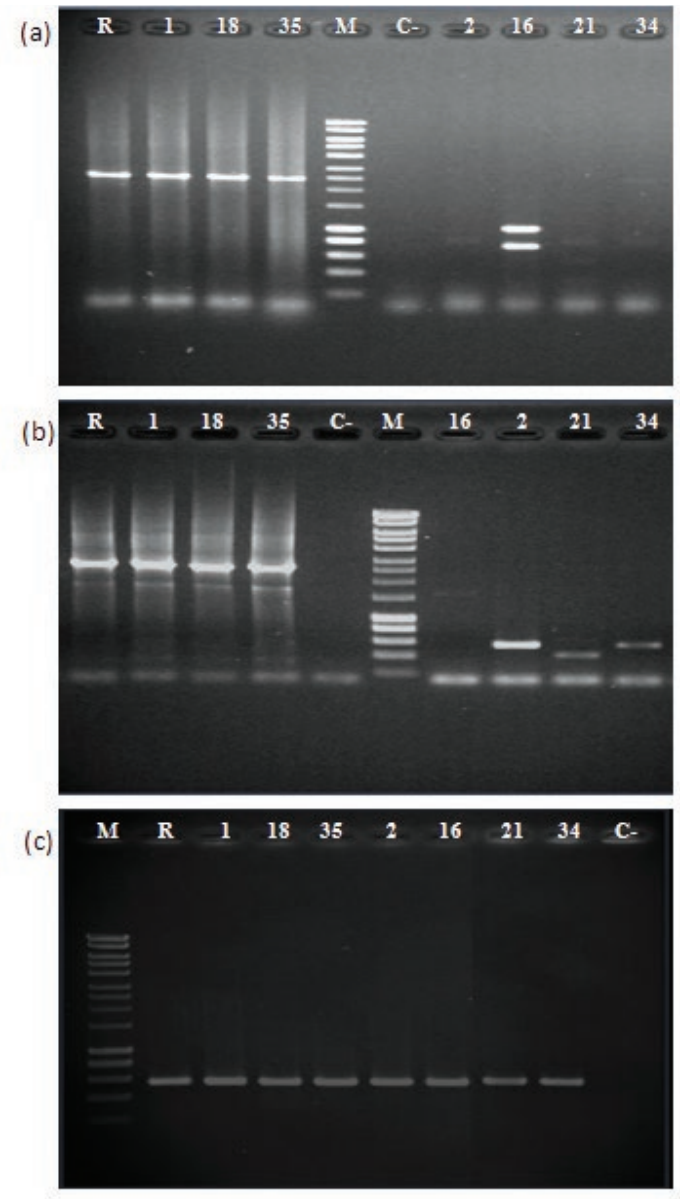

Fig. 2. PCR analysis of Tunisian *E.amylovora* strains with CRISPR primers. (a) CRISPR 1; (b) CRISPR 2; (c) CRISPR 3. C, negative control. M, size marker (1kb DNA ladder, Invitrogen). R, reference strain *E. amylovora* CFBP 1430.

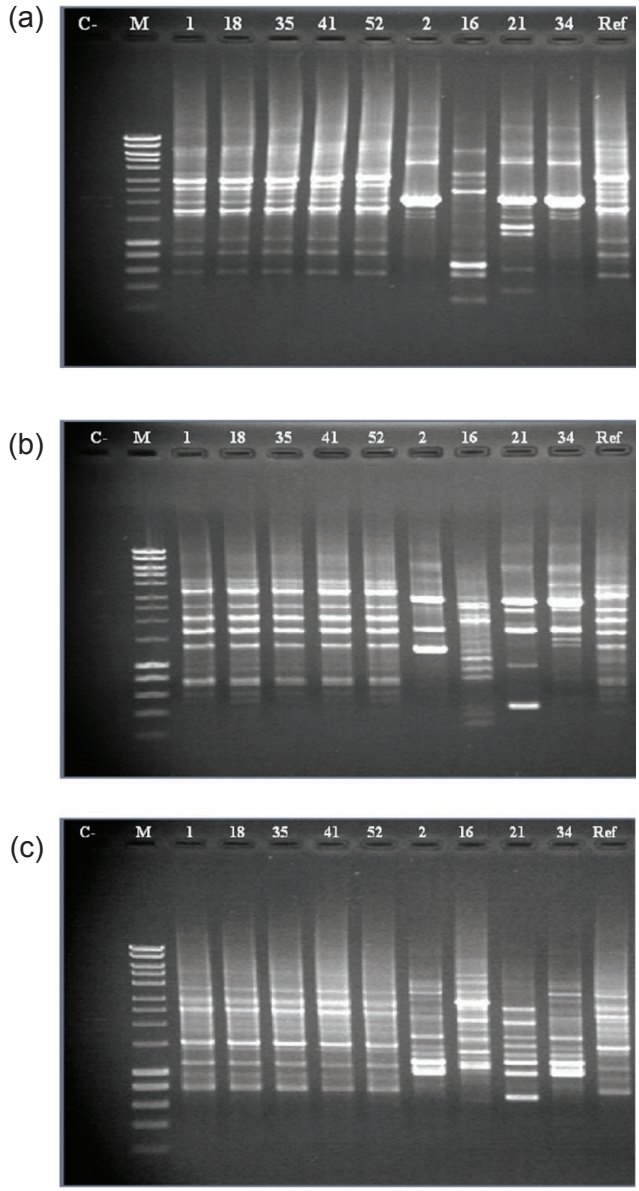

Fig. 3. Typical and specific profiles of the Tunisian *E. amylovora* strains obtained with the three VNTR primers and comparison of the Tunisian isolates with the European reference strain CFBP 1430 (Ref). (a) Amplification obtained with VNTR 4; (b) Amplification obtained with VNTR 5; (c) Amplification obtained with VNTR 6. M, Size marker (1kb DNA ladder, Invitrogen). C-, negative control.

previously detected among CRISPR array sequences (McGhee and Sundin, 2012) and the potential presence of two CRISPR 1 loci in certain *E. amylovora* isolates. With CRISPR 2, there was no amplicon for strain 16, the same amplicon size for strains 2 and 34 and a lower size amplicon for strain 21 (Fig. 2b). In addition, strains 2 and 34 showed the same amplicon size with CRISPR 2, leading us to suggest that they are genetically closely related.

As it was expected from the high conservation of CRISPR 3 array, all strains showed identical amplification with primers bordering this array (Table 1; Fig. 2c). Based on combination of the obtained patterns for CRISPR spacer arrays 1, 2, and 3, we identified 4 distinct genotypes (Table 1). Similarly, 18 different genotypes were found within a collection of 37 cosmopolitan *E. amylovora* strains (Rezzonico *et al*., 2011). These results were not surprising, since it was already demonstrated that subtyping using CRISPRs can be a robust tool for differentiating genotypes of many bacteria species such as *Mycobacterium tuberculosis* (Groenen *et al*., 1993), *Yersinia pestis* (Pourcel *et al*., 2005), *Salmonella enterica* (Fabre *et al*., 2012) and *E. amylovora* (McGhee and Sundin, 2012; Shariat and Dudley, 2014).

Analysis of the VNTR(s) fingerprinting profiles. Fifty-three Tunisian isolates of *E. amylovora* from different regions and plant hosts were evaluated for VNTR sequences (Table 1). Each VNTR primer generated a specific band

Table 1. Origin and molecular characterization of the Tunisian *E. amylovora* strains used in this study.

Year	Isolates	Origin	Host/Variety	*Specific PCR Amplifications	PCR fingerprinting profiles					
					CRISPR-1	CRISPR-2	CRISPR-3	VNTR-4	VNTR-5	VNTR-6
2012	13	Mornague	Pear, Apple	+	A	B	C	D	E	F
	1	Bejaoua	Pear/ Bouguedma	+	A	B	C	D	E	F
	N°34 (SC200)[α]	**Mornague**	**Pear/ Williams**	**+**	**–**	**B1**	**C**	**D1**	**E4**	**F1**
2013	20	Ariana, Ben Arous, Bizerte, Manouba	Pear, Apple	+	A	B	C	D	E	F
	N°2 (SC159)[α]	**Mornague**	**Apple/ Golden**	**+**	**–**	**B1**	**C**	**D1**	**E1**	**F1**
	N°16 (SC174)[α]	**Naassan**	**Pear/ Williams**	**+**	**A1**	**–**	**C**	**D2**	**E2**	**F2**
	N°21 (SC179)[α]	**Khelidia**	**Apple/Anna**	**+**	**–**	**B2**	**C**	**D3**	**E3**	**F3**
2014	17	Ariana, Ben Arous, Bizerte, Manouba	Pear, Apple	+	A	B	C	D	E	F
Reference type strain : *E. amylovora* CFBP 1430				+	A	B	C	D	E	F

(*) Specific *E. amylovora* primers (AJ75/AJ76; G1-F/G2-R; FER1-F/gER2R; ErwRpoB-F/ErwRpoB-R and EAPSGL3961/EAPSGL4610c). (**α**): Correspondent number of the strain in the GenBank database. (+): positive amplification; (–): no amplification. A, B and C: identical profiles obtained with the three CRISPRs. A1, B1 and B2: different profiles obtained with the three CRISPRs. D, E and F: identical profiles obtained with the three VNTRs. E1, F1 and F2: different profiles obtained with the three VNTRs.

pattern. The results indicated the emergency of a major group of homogeneous strains and the existence of several polymorphic bands in the *E amylovora* strains studied in this work. Remarkably, for all used VNTR sequences, the largest group (49 strains) showed identical patterns as the reference strain CFBP 1430. Likewise, four strains consistently showed distinct patterns (strains 2, 16, 21 and 34) (Table 1; Fig. 3). Indeed, VNTR 4 allowed us to distinguish four different genotypes (Fig. 3a): a preponderant genotype (49 strains) and three completely different genotypes (2 and 34 with identical profile, as well as 16 and 21). Profiles obtained with VNTR 6 reproduced roughly the same results (Fig. 3c). Nevertheless, profiles obtained with VNTR 5 indicated five distinct genotypes: all isolates displayed the same fingerprinting profile except isolates 2, 16, 21 and 34, which were completely different (Fig. 3b). Thus, we could confirm that strains 2 and 34 have different genotypes and that five different genotypes exist within the Tunisian collection of *E. amylovora*.

Several methods such as RFLP and AFLP used in epidemiological studies of *E. amylovora* have shown that there is a low genetic variability within this species. Regarding our VNTR analysis, however, we noticed a degree of genetic variability among Tunisian isolates. Previous studies have detected two distinct patterns by VNTR sequences (Hannou *et al.,* 2013) on the Moroccan collection of *E. amylovora*. Moreover, a recent molecular work based on Multiple-Locus VNTR analysis (MLVA) allowed the distinction of 227 haplotypes among a collection of 833 isolates of *E. amylovora* of worldwide origin (Bühlmann *et al*., 2014).

Based on its success with other bacterial species such as *Mycobacterium tuberculosis* (Mazars *et al*., 2001), *Yersinia pestis* (Klevytska *et al*., 2001) and *Staphylococcus aureus* (Malachowa *et al*., 2005), the VNTR technique proved to be highly valuable in ecological, epidemiological and evolutionary studies, aiming at investigating the genetic variability of *E. amylovora* worldwide and also now in Tunisia.

When looking for diversity among Tunisian strains by CRISPR loci and VNTR analysis, we determined five distinct PCR fingerprints: (i) the major group (49 strains), (ii) strain 2, (iii) strain 16, (iv) strain 21 and (v) strain 34. Strains 2 and 34, isolated from relatively young pear trees in the Mornague region, appear to be genetically related since they produced identical fingerprints with the CRISPR and VNTR primers, except with VNTR5 (Table 1). Conversely, the two other divergent isolates (16 and 21), obtained from apple trees in Khelidia and Mornague, respectively, showed distinctive band patterns with all the analyzed primers. Despite these differences, these four strains (2, 16, 21 and 34) were confirmed as *E. amylovora* by biochemical, molecular and pathogenicity characteristics.

In conclusion, our results possibly suggest that there have been multiple introductions of *E. amylovora* into Tunisia, although we can not discount the possibility that there has been a unique introduction with trees infected with a genetically diverse population of the pathogen or that mutations occurred after its introduction. The current hypothesis is that the disease originated from the introduction of plant material from other countries. Nevertheless, the introduction of fire blight into Tunisia could probably be better reconstructed should we have more information about the source of infected plants or the nursery providing them.

In summary, this study describes the first molecular characterization of Tunisian *E. amylovora* isolates. The results revealed that all Tunisian isolates produced the expected amplicons with diverse primer pairs routinely used for molecular diagnostics of *E. amylovora* and consequently confirmed their inclusion in this species. We also

recorded some genetic variability among this collection by using PCR fingerprinting (CRISPR and VNTR). Moreover, we found that out of all molecular techniques used, those developed on the basis of released pan-genome *E. amylovora* sequences, VNTR and CRISPR, seem to be the most promising in differentiation of the fire blight pathogen and that VNTR analysis seems to be more efficient in epidemiological and source tracking investigation of *E. amylovora.*

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