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SHORT NOTES

Molecular characteristics of a strain (Salento-1) of *Xylella fastidiosa* isolated in Apulia (Italy) from an olive plant with the quick decline syndrome

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Summary. DNA-based approaches were used to characterize a strain (Salento-1) of *Xylella fastidiosa* obtained from an olive plant suffering from the syndrome of quick decline in Apulia (South Italy). Salento-1 was indistinguishable from strain CoDiRO previously isolated from olive in Apulia and assigned to *X. fastidiosa* subsp. *pauca.* Based on our results and comparative analysis with reported data, the subspecies *pauca*, *multiplex*, and *fastidiosa* may invade olive throughout the world (California, Italy, Argentina and Brazil). The strain Salento-1 has been deposited in the National Collection of Plant Pathogenic Bacteria (NCPPB), England, and in the Belgian Coordinated Collections of Microorganisms (BCCM), Belgium.

Key words: olive decline, MLST, *dnaA*, *rpoD*, intergenic region, genetic relationships.

Introduction

Xylella fastidiosa (Wells *et al*., 1987) is a Gram negative fastidious bacterium known to infect many plant species, including grape, almond, plum, peach, oak, citrus and coffee (Janse and Obradovic, 2010; Purcell, 2013), mainly in warm climate regions of the Americas.

Although the host range of *X. fastidiosa* continues to expand, there is evidence that selected strains of the pathogen induce diseases only in specific hosts, causing symptomless infections on other hosts (Purcell, 2013). To date, only two *X. fastidiosa* subspecies [(*fastidiosa* (Wells *et al*. 1987) and *multiplex* (Schaad *et al*. 2009)] have been validly published (Euzéby, 2009; Bull *et al*., 2012). Two more subspecies (*pauca* and *sandyi*) have been described, but not yet published according to certain rules of the International Code of Nomenclature of Bacteria (Schaad *et al*., 2004; Schuenzel *et al*., 2005; EFSA, 2015) (Rule 30 in the case of subsp. *pauca*: Bull *et al*., 2010). As well, two other subspecies (*tashke* and *morus*), respectively proposed by Randall *et al*. (2009) and by Nunney *et al*. (2014a), are awaiting confirmation.

Olea europea, which has been recently added to the list of *X. fastidiosa* hosts, was found infected by *X. fastidiosa* subsp. *multiplex* in Southern California (Hernandez-Martinez *et al*., 2007; Krugner *et al*., 2014). In 2013, the presence of the *X. fastidiosa* DNA

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was reported from Italy, on almond, oleander and olive plants. Olive plants were affected by a decline syndrome that had spread in the Salento peninsula of Apulia over an area of 8,000 ha (Saponari *et al*., 2013; Cariddi *et al*., 2014; Lo Console *et al*., 2014). Affected plants exhibited leaf scorch symptoms, galleries in trunks and branches made by larval stages of *Zeuzera pyrina* (leopard moth), and extended discoloration of the wood from which different fungi were isolated (Nigro *et al*., 2013; Cariddi *et al*., 2014). The outbreak of *X. fastidiosa* is now extended to almost the whole Lecce province, and has reached the nearby Brindisi province (Figure 1; EFSA, 2015; Bollettino Ufficiale della Regione Puglia, 2015). In Apulia (southern Italy), *X. fastidiosa* has been detected from 20 plant species other than olive, but its etiological role in olive decline is yet to be determined (http://cartografia.sit.puglia.it/doc/Gli_Ulivi_Pugliesi_Oltre_la_Xylella.pdf; EFSA, 2015). Based on Multi Locus Sequence Typing (MLST) analysis, Xf9, the first Apulian strain to be isolated from an olive plant showing leaf scorch and die-back symptoms (Elbeaino *et al*., 2014), was reported to be phylogenetically related to *X. fastidiosa* subsp. *pauca* 9a5c (Simpson *et al*., 2000). Moreover, strain Xf9 was shown to have the same allelic profile (ST53) as some atypical strains of *X. fastidiosa*

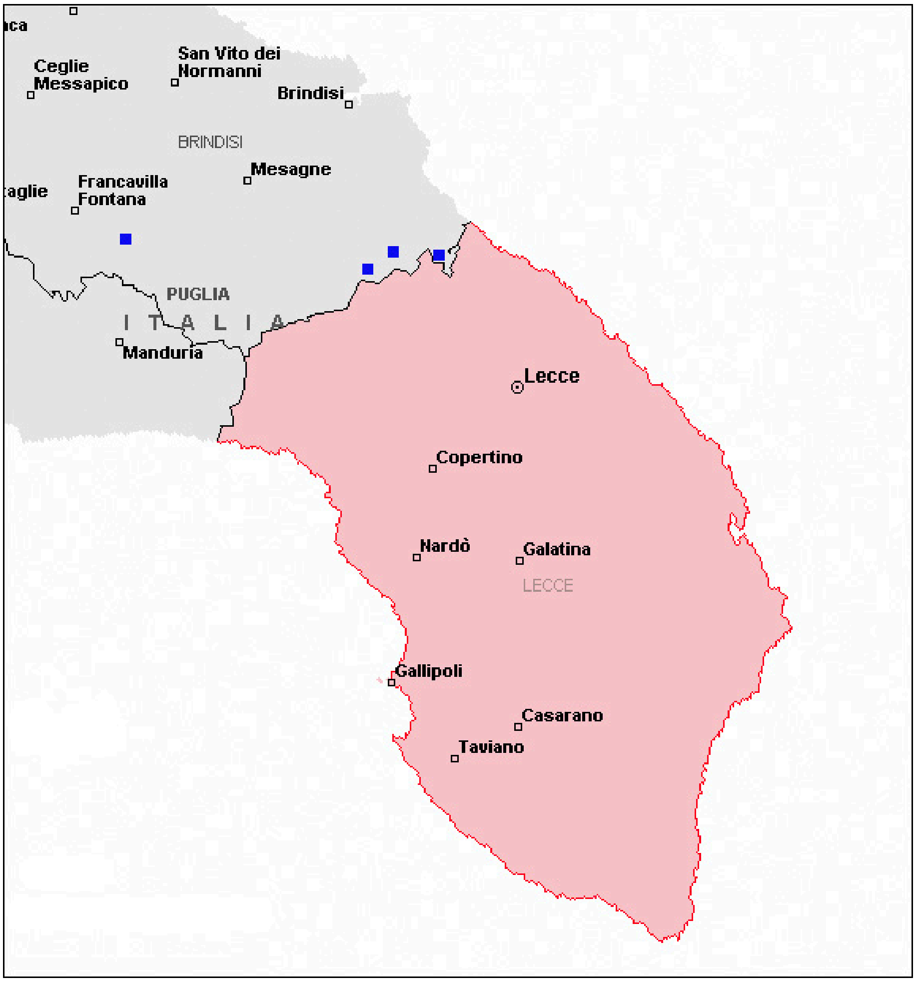

Figure 1. Distribution of *Xylella fastidiosa* in the Apulia region (Italy). The main outbreak, corresponding to the whole of the Lecce province, is highlighted in pink, while the outbreaks recorded at Oria, S. Pietro Vernotico, Cellino S. Marco and Torchiarolo in the Brindisi province are highlighted in blue. The map was redrawn and adapted from Bollettino Ufficiale della Regione Puglia, 2015.

subsp. *pauca* that were isolated in Costa Rica from coffee and oleander (Nunney *et al*., 2014b). The disease of olive observed in the Salento peninsula was first named "Complex of the quick decline syndrome" (CoDiRO) (Guario *et al*., 2013) and soon after changed to "olive quick decline syndrome" (OQDS) (Cariddi *et al*., 2014). However, the acronym CoDiRO is still maintained and used to designate the disease as well as the first strain of *X. fastidiosa* from olive whose genome has been sequenced (Giampetruzzi *et al*., 2015; Martelli *et al*., 2016). *Xylella fastidiosa* subsp. *pauca* was also detected by molecular methods in Argentina in 2015, in olive plants showing symptoms described as decay, desiccated or dull green colored leaves, and death of shoots and branches (Haelterman *et al*., 2015). More recently, *X. fastidiosa* subsp. *pauca* has also been isolated from olive plants in Brazil (Della Coletta Filho *et al*., 2016).

Here, we report the molecular characterization of *X. fastidiosa* strain Salento-1, which was isolated in 2015 from an olive plant affected by severe decline at Taviano, Lecce province. The strain is referred as "strain 5" in the collection of Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche in Lecce, Italy.

Materials and methods

Sampling, DNA extraction and isolation

In early 2015, an olive orchard located in Taviano was inspected to identify trees displaying symptoms of OQDS. Samples of 1-year-old twigs (cv. Ogliarola salentina) with dull green leaves were randomly collected from the canopies of each of six symptomatic plants and immediately transported to the laboratory. To verify presence of *X. fastidiosa*, five to seven leaves from each plant were thoroughly washed under running tap water, air-dried and the entire length of their mid-ribs and petioles were excised with a sterile scalpel. Tissues were ground in liquid nitrogen with mortar and pestle and total DNA was extracted using the DNeasy plant mini kit (Qiagen). Presence of *X. fastidiosa* DNA in leaf samples was determined using the PCR primer set FXYgyr499/RXYgyr907 and conditions described by Rodrigues *et al.* (2003). For bacterial isolation, twigs were rinsed in tap water, cut to approx. 10–15 cm length, disinfected in 2% sodium hypochlorite for 3 min, followed by rinsing in 70% ethanol for 2 min, and three rinses in sterile distilled water, and drying in a laminar flow hood. Sap was directly squeezed out from the twigs with sterile pliers and blotted directly onto buffered charcoal-yeast extract (BCYE, Lamb) agar plates (Wells *et al*., 1981). Isolation plates were sealed with parafilm M (Bemis) and incubated at 28°C. Colonies that exhibited fastidious growth were selected, suspended in 100 μL of sterile distilled water, tested for purity on BCYE agar plates, and their identity was then confirmed by PCR screening using the protocol described above. PCR-positive colonies were stored at -80°C in sterile PBS buffer (Sigma) containing 50% glycerol (Sigma).

Molecular identification

Genomic DNA of a selected olive-infecting strain was extracted with the GenElute Mammalian Genomic Minipreps Kit (Sigma) following manufacturer's instructions, and used as the template for PCR amplification of a 733 bp fragment using the *X. fastidiosa* specific primers RST33, located near the 3' end of the *rpoD* gene, and RST31, located in the downstream intergenic region (Minsavage *et al*., 1994; Chen *et al*., 2005). To perform MLST analysis, a fragment of each of seven housekeeping genes (*cysG*, *gltT*, *holC*, *malF*, *leuA*, *nuoL*, and *petC*) was amplified according to the procedure described by Yuan *et al*. (2010). Concentrations of reagents in the PCR mix, as well as the cycling conditions, were as originally described. However, in our assay we used the Dream Taq Green DNA Polymerase (Thermo-Scientific) and increased the annealing temperature of the primers for the *malF* gene to 68°C. Two new primer sets Xfa-rpod-F4/R4 (5'-ACTGAGGTTGTCGTTGGCTT-3'/5'-CCTCAGGCATGTCCATTTCC-3') and Xfa-dnaA-2F/2R (5'-TTCCATCAAATTGACGCGCT-3'/5'-CGGCAAGCATGTAACACTGT-3') were designed based on sequence comparison from the genomes of *X. fastidiosa* subsp. *pauca* 9a5c (GenBank accession No. NC_002488.3), *X. fastidiosa* subsp. *multiplex* M12 (GenBank accession No. NC_010513.1), and *X. fastidiosa* subsp. *fastidiosa* M23 (GenBank accession No. NC_010577.1). These were used to amplify, respectively, a 988 bp portion of the RNA polymerase sigma-70 factor (*rpoD*) and a 650 bp portion of the chromosomal replication initiator protein DnaA (*dnaA*) genes. Each reaction mixture for amplification contained 1 × DreamTaq Green Buffer (Thermo Scientific), 0.2 mM each dNTP, 0.4 μM each primer, 1 U Dream *Taq* Green DNA Polymerase (Thermo Scientific), approx. 5 ng of template DNA and DNase free water to a final volume of 25 μL. The amplification of both genes was carried out in the same cycling conditions: after a denaturation step of 5 min at 94°C, a total of 35 cycles were performed (30 s at 94°C for denaturation, 1 min at 61°C for annealing, 30 s at 72°C for extension). DNA of both *X. fastidiosa* subsp. *fastidiosa* (M23) and *X. fastidiosa* subsp. *multiplex* (M12) reference strains was always used as control. All PCR products were visualized after electrophoresis in 1 or 2% agarose gels in 1 × Tris–acetate–EDTA (TAE) buffer and staining with ethidium bromide (0.5 μg mL^{-1}). They were purified using ExoSAP-IT (USB-Affymetrix), and both strands were sequenced on an ABI prism 3130 Genetic Analyzer system (Applied Biosystems). Sense and antisense nucleotide sequences were visualized and checked for quality using CHROMAS LITE 2.01 (Technelysium), aligned using MUSCLE as implemented in MEGA6 (Tamura *et al*., 2013), and single consensus sequences were determined.

Analysis of sequence data

After removal of primer oligonucleotides, identity searches were performed on the INSDC database (http://www.insdc.org/). For MLST data, searches were carried out on the *X. fastidiosa* MLST databases website (http://pubmlst.org/xfastidiosa), to determine the allele numbers and the resulting Sequence Type (ST). For *rpoD* and *dnaA* gene fragments, nucleotide sequences alignments including the corresponding alleles of 19 strains of *X. fastidiosa* whose complete or draft genomes are publicly available (http://www.insdc.org/), were used to construct dendrograms in MEGA6 by using the Kimura 2 parameters distance and the neighbour joining method. Confidence levels of the branching points were determined using 1000 bootstrap replicates. *Xylella fastidiosa* Salento-1 sequences were registered in the INSDC GeneBank under accession numbers KU214450 to KU214457, KU297284, and KU297285.

Results and discussion

The survey carried out in an olive orchard in Taviano revealed that nearly 80% of the plants showed symptoms of the OQDS. Amplification of DNA extracted from dull green leaves of 1-year-old twigs (Figure 2) by primers FXYgyr499/RXYgyr907 produced an amplicon of the expected size (approx. 429 bp). This corresponds to the *gyrB* gene of *X. fastidiosa* according to the sequencing analysis results (data not shown). Twenty-five d after incubation on BCYE agar, PCR screening results indicated that *X. fastidiosa* had been cultured from the twigs of the tested plants. One isolate, Salento-1, was subjected to colony purification and further molecular characterization. Purified colonies of *X. fastidiosa* Salento-1 grown on BCYE agar for 20 d at 28°C were slightly convex, white, opalescent, mucoid when touched with a loop, circular with entire margins, with a diameter of about 1.2–1.5 mm and a smooth surface (Figure 3). These morphological characteristics were maintained through at least seven serial subcultures over a period of 6 months. MLST housekeeping gene fragment analysis and *X. fastidiosa* MLST database querying results unambiguously showed that *X. fastidiosa* Salento-1 strain shares the same sequence type (ST) 53 with the previously described strains of *X. fastidiosa* subsp. *pauca* Xf9 (Elbeaino *et al*., 2014) and CoDiRO (Giampetruzzi *et al*., 2015), which were isolated from olive plants in Apulia. To date, six *X. fastidiosa* strains isolated in Costa Rica, from coffee (one strain) and oleander (five strains), as well as six strains isolated in Apulia, from olive (three strains), oleander (one strain), almond

Figure 2. Symptoms of the quick decline syndrome on 1-year-old twigs of olive plants in Taviano (Lecce, South Italy). Dull green leaves were sampled for *Xylella fastidiosa* DNA detection.

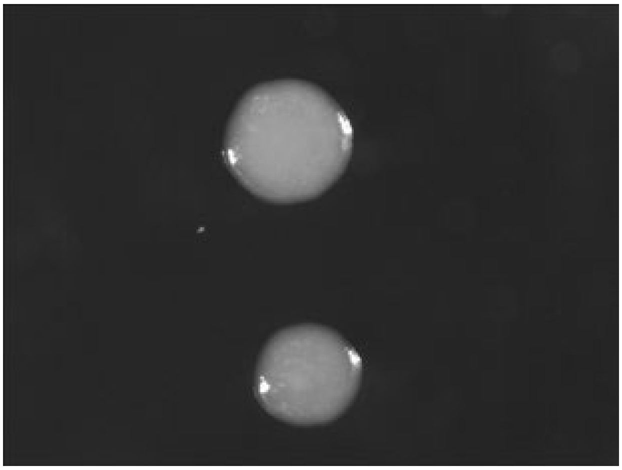

Figure 3. Colonies of *Xylella fastidiosa* subsp. *pauca* Salento-1, after incubation at 28°C for 20 d on BCYE agar plates.

(one strain) and periwinkle (one strain), have been typed as ST53, according to the MLST database records (http://pubmlst.org/xfastidiosa). Nucleotide sequences of 610 and 1308 bp were obtained, respectively, for the *dnaA* and *rpoD* genes. Both sequences were 100% identical to those of the *X. fastidiosa* subsp. *pauca* CoDiRO strain isolated in Apulia. According to neighbour joining analysis of the *dnaA* fragment, strains Salento-1 and CoDiRO could not be distinguished from two (9a5c and 32) out of three *X. fastidiosa* subsp. *pauca* strains that were considered for comparison (Figure 4). However, the same analysis carried out for the *rpoD* gene clearly separated the two Apulian strains from the *X. fastidiosa* subsp. *pauca* strains isolated from citrus (9a5c) and coffee (6c and 32) in Brazil (Figure 5). When a portion of the intergenic region located between the 3′ end of gene *rpoD* and upstream of primer RST31 (Minsavage *et al*., 1994) of strain Salento-1 was aligned with the corresponding region of eight *X. fastidiosa* subsp. *pauca* strains, two different sequences were evident according to the presence of 3 SNP's and of two indels of 4 and 14 bp, respectively (Figure 6). Strains Salento-1, CoDiRO and Xf9 isolated in Apulia from olive share an identical 186 nucleotide sequence, whereas three

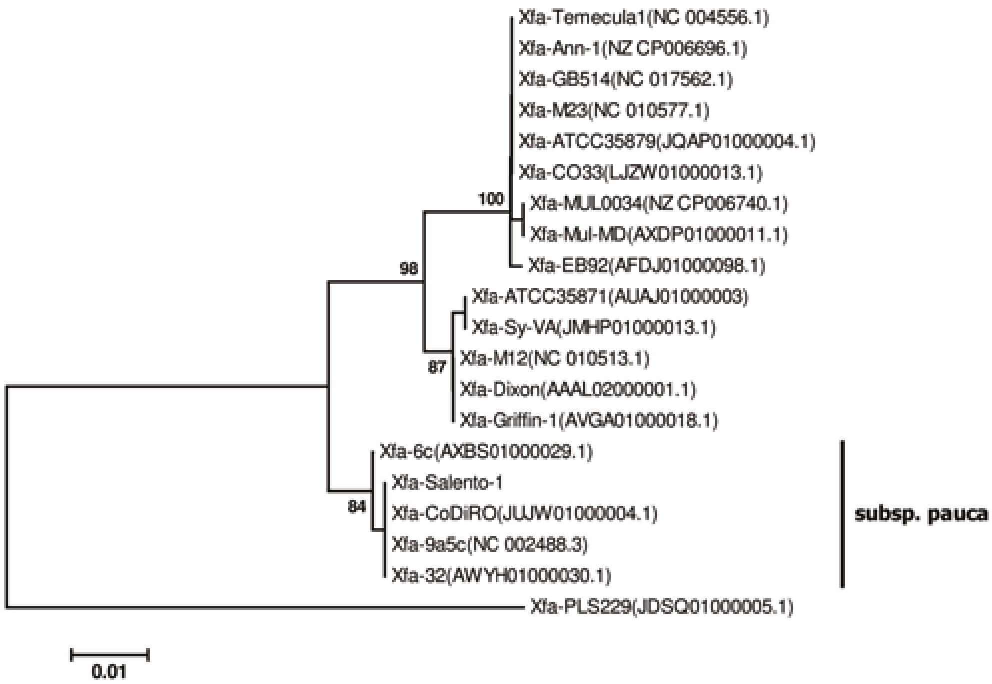

Figure 4. Neighbour joining dendrogram showing the similarity of 20 strains of *Xylella fastidiosa* based on *dnaA* partial sequence analysis (610 nucleotides). Numbers above or below branches are bootstrap values based on 1000 pseudoreplicates (only values above 80% are shown) under the Kimura 2 parameters model of evolution with gamma rate of variation across sites. The accession numbers of the reference strains genomes are given in parenthesis.

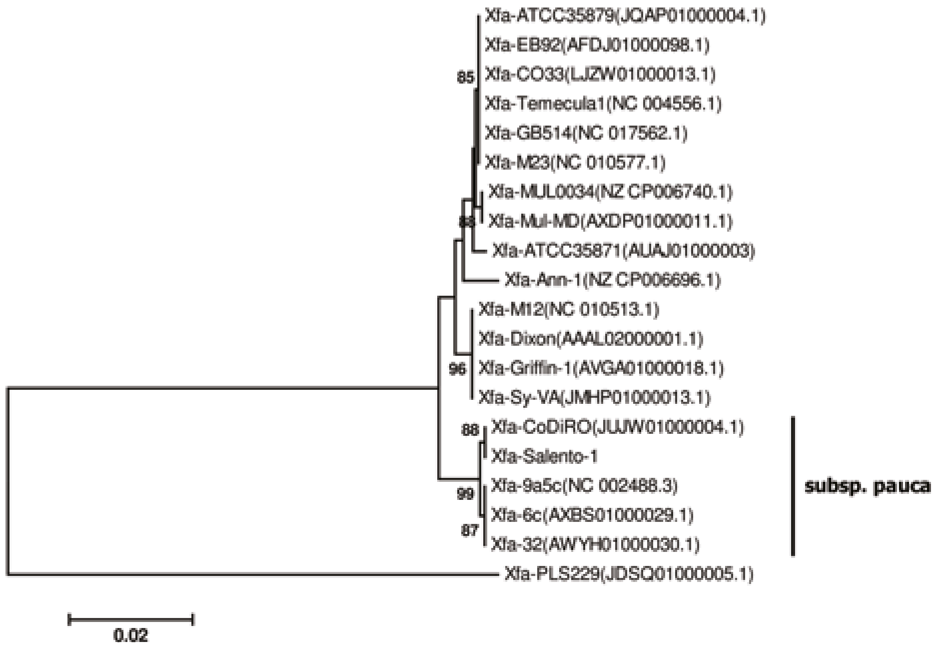

Figure 5. Neighbour joining dendrogram showing the similarity of 20 strains of *Xylella fastidiosa* based on *rpoD* partial sequence analysis (1308 nucleotides). Numbers above or below branches are bootstrap values based on 1000 pseudoreplicates (only values above 80% are shown) under the Kimura 2 parameters model of evolution with gamma rate of variation across sites. The accession numbers of the reference strains genomes are given in parenthesis.

strains of subsp. *pauca* isolated in Brazil (9a5c, 6c and 32) and three uncultured *X. fastidiosa* strains (CE1, CE2 and AM1) detected in olive trees affected by decline in Argentina (Haelterman *et al*., 2015) share an identical sequence of 204 bp. The usefulness of this locus to differentiate *X. fastidiosa* genotypes has been previously described (Chen *et al*., 2005). Among other differences, the existence of a repeat sequence of two units of 14 bp in *X. fastidiosa* subsp. *pauca* 9a5c was absent in *X. fastidiosa* subsp. *fastidiosa* Temecula 1, subsp. *multiplex* Dixon1 and subsp. *sandyi* Ann1. We have found that this repeat occurs in subsp. *pauca* strains other than 9a5c, but it is absent in the Apulian strains from olive (Figure 6).

Olive plants showing leaf scorching symptoms in Brazil were recently found to be infected by a strain of *X. fastidiosa* subsp. *pauca* with a sequence type-ST16, thus different from the strain detected in Apulia (Della Coletta Filho *et al*., 2016). Therefore, based on current evidence, different strains of *X. fastidiosa* (subsp. *pauca*, *multiplex*, and *fastidiosa*) may infect olive. It is also likely that some genetic heterogenicity could exist in the Apulian population of *X. fastidiosa* (Elbeaino *et al*., 2014).

The assignation of the strains CoDiRO, Xf9 and Salento-1 to the subspecies *pauca* is a contradiction in terms from a nomenclatural point of view. The Latin meaning of *pauca* is, little, few, and implies the narrow host range origins of the *X. fastidiosa* populations assigned to the subspecies (Schaad *et al*., 2004). Instead, it seems that the *X. fastidiosa* populations found in Salento are able to infect (here and elsewhere the term “infected” is not used as synonymous of disease) several hosts in numerous plant families (Martelli *et al*., 2015). This issue requires further and deeper study, and the opportunity to use different names for the bacterial populations found in the Salento region is not excluded.

Xylella fastidiosa strain Salento-1 has been deposited in the National Collection of Plant Pathogenic Bacteria (NCPPB) and in the Belgian Coordinated Collections of Microorganisms (BCCM).

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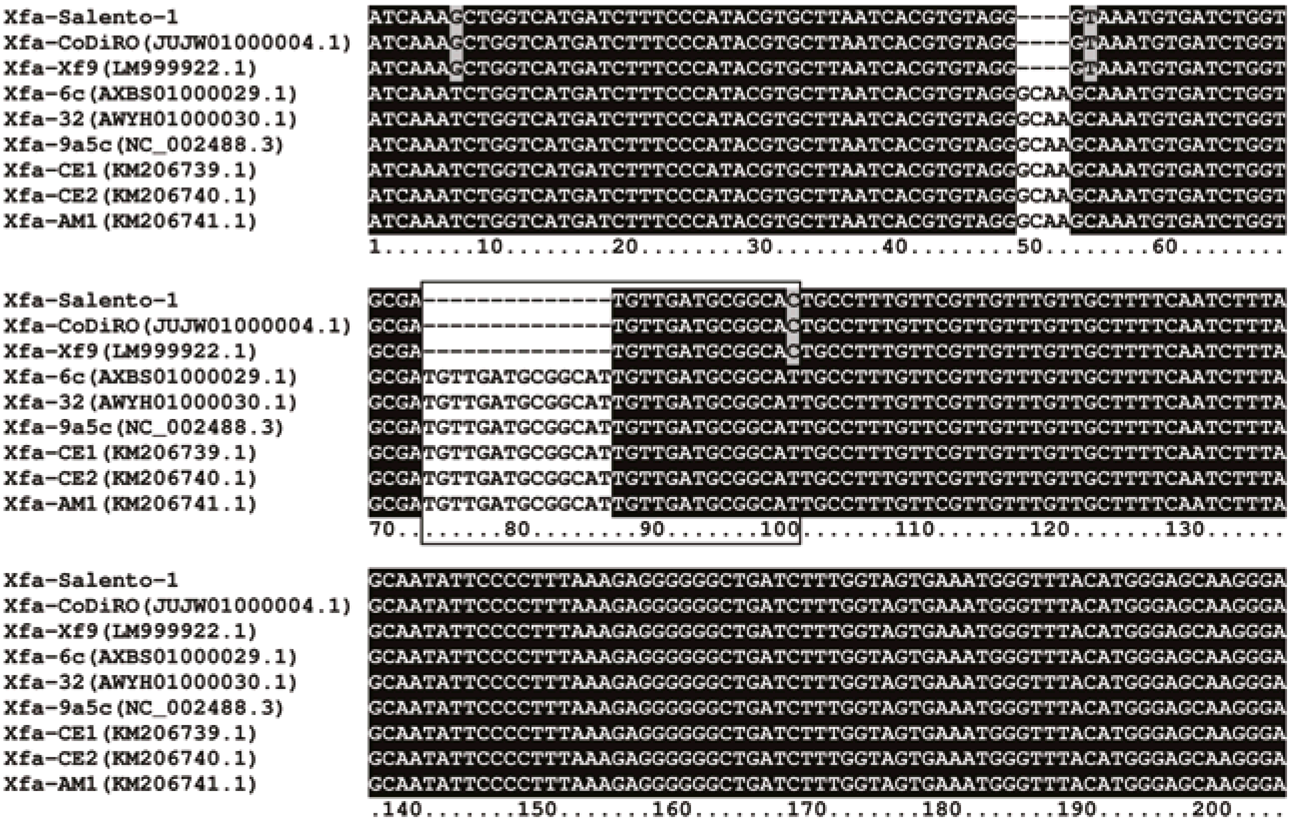

Figure 6. Alignment of a fragment of the intergenic region downstream the 3′ end of *rpoD* from nine *Xylella fastidiosa* subsp. *pauca* strains. Strains Salento-1, CoDiRO and Xf9 were isolated in Apulia from olives affected by the quick decline syndrome; strains 9a5c (citrus), 6c and 32 (coffee) were isolated in Brazil; uncultured *Xylella* strains CE1, CE2 and AM1 were detected in olive plants in Argentina. SNP's and indels are highlighted, respectively, in gray and white. The repeat sequence of two 14 bp units reported as characteristic of strain 9a5c (Chen *et al*., 2005) is boxed. The figure was drawn using BOXSHADE (http://mobyle.pasteur.fr).

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