Phytopathologia Mediterranea (2014) 53, 3, 533–542
DOI: 10.14601/Phytopathol_Mediterr-15000

RESEARCH PAPERS

Multilocus sequence typing of *Xylella fastidiosa* isolated from olive affected by "olive quick decline syndrome" in Italy

Toufic ELBEAINO, Franco VALENTINI, Raied ABOU KUBAA, Peter MOUBARAK, Thaer YASEEN and Michele DIGIARO

CIHEAM - Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy

Summary. The recent finding of *Xylella fastidiosa* (*Xf*) in olive trees in southern Italy, the scanty molecular information on this bacterium and its association with the olive quick decline syndrome (OQDS) prompted the necessity to isolate and acquire more genetic data on the type of strain present in that region. For the first time, the bacterium was isolated from infected olive on culture media. Genetic information were obtained through genomic comparison with other subspecies or strains. The sequences of thirteen genes from its genome, comprising seven housekeeping genes (*leuA, petC, lacF, cysG, holC, nuoL* and *gltT*) usually used in multilocus sequence typing (MLST) systems, and six genes involved in different biochemical functions (*RNA Pol sigma-70 factor, hypothetical protein HL, 16S rRNA, rfbD, nuoN,* and *pilU*), were analyzed. The sequences of the biochemical function genes were explored individually to study the genetic structure of this bacterium, while the MLST genes were linked together into one concatameric sequence (4161 bp long) to increase the resolution of the phylogenetic analysis when compared with *Xf* strains previously reported. Sequence analyses of single genes showed that the *Xf* olive strain is distinct from the four previously defined taxons (*Xf* subsp. *fastidiosa*, *Xf* subsp. *multiplex*, *Xf* subsp. *sandyi* and *Xf* subsp. *pauca*) with a dissimilarity rate that reached 4%. In particular, *Xf* from olive shared the greatest identity with the strain "9a5c" (subsp. *pauca*), but was nevertheless distinct from it. Similarly, the MLST based on concatameric sequences confirmed the genetic variance of *Xf* from olive by generating a novel sequence type profile (ST53). Phylogenetic tree analyses showed that *Xf* from olive clustered in one clade close to subspecies *pauca* (strains "9a5c" and "CVC0018"), but was nevertheless distinct from them. These results indicate molecular divergence of this olive bacterium with all other strains yet reported.

Key words: isolation, PCR, MLST, phylogenetic analyses.

Introduction

Xylella fastidiosa (*Xf*) is a Gram-negative, xylem-limited, plant pathogenic bacterium (Wells *et al*., 1987). Diseases caused by *Xf* include Pierce's disease on grapes (Davis *et al*., 1978), citrus variegated chlorosis (CVC) (Chang *et al*., 1993), coffee leaf scorch (Li *et al*., 2001), pecan leaf scorch (Sanderlin and Heyderich-Alger, 2000), phony peach disease (Wells *et al*., 1983), plum leaf scald (Raju *et al*., 1982) and almond leaf scorch (Mircetich *et al*., 1976). *Xylella fastidiosa* has also been shown to be the causal agent of diseases found in landscape plants such as oleander leaf scorch (Purcell *et al*., 1999), mulberry leaf scorch (Hernandez-Martinez *et al*., 2006) and oak leaf scorch (Barnard *et al*., 1998). In addition to these proven *Xf*-disease relationships through completion of Koch's postulates, *Xf* is known to be associated with leaf scorch symptoms in olive and other ornamental landscape species including crape myrtle, day lily and southern magnolia (Hernandez-Martinez *et al*., 2006).

Four subspecies of *Xf* have been identified (Schaad *et al*., 2004; Schuenzel *et al*., 2005), each showing a high degree of host specificity. *Xylella fastidiosa* subspecies *fastidiosa* is responsible for Pierce's disease of grapevine (Buzombo *et al*., 2006), but also causes

Corresponding author: T. Elbeaino
E-mail: elbeaino@iamb.it

ISSN (print): 0031-9465
ISSN (online): 1593-2095

disease in almond, alfalfa, and, in Central America, coffee (Nunney *et al*., 2010). The subspecies *sandyi* is largely restricted to oleander (Yuan *et al*., 2010), while subsp. *multiplex* infects almond (like subsp. *fastidiosa*), but also other related fruit trees including plum, peach and apricot. In addition, subsp. *multiplex* infects a range of native trees, including oak and sweetgum (Hernandez-Martinez *et al*., 2006; Schaad *et al*., 2004; Schuenzel *et al*., 2005). The fourth subspecies, subsp. *pauca*, is present only in South America where it is considered a major threat to the citrus industry and to coffee, on which it induces leaf scorch symptoms (Nunney *et al*., 2010). The subdivision of *Xf* into four subspecies, three of which are found in the United States, and the evidence of recombinant events among the subspecies (Scally *et al*., 2005; Nunney *et al*., 2012) make the study of genetic characterization of *Xf* isolates very important. In California, *Xf* was isolated from olive, shown to be pathogenic to oleander and almond but not to grapevine, and recognized as a member of subsp. *multiplex* (Krugner *et al*., 2014).

In the Apulia region of Southern Italy, *Xf* was recently reported in association with a severe disease of olive named "olive rapid decline complex, CoDiRO" (Saponari *et al*., 2013) or "olive quick decline syndrome" (OQDS; Cariddi *et al*., 2014). The pathogen was found to be transmitted by the meadow spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae) (Saponari *et al*., 2014), while *Neophilaenus campestris* Fallén (Aphrophoridae) and *Euscelis lineolatus* Brullé (Cicadellidae) were reported to be additional potential vectors (Elbeaino *et al*., 2014). The presence of this bacterium in that area has emerged as a potential threat to olive production, not only for Italy, but also for the rest of Europe and the Mediterranean basin.

The accentuated severity of the disease and the quick expansion in large olive growing areas has prompted the necessity of a critical study on host specialization, disease development and genome characterization of the bacterial strains involved in the disease. Such genetic and biological information could only be acquired through genomic comparison with other strains from different sources already known. Numerous serological techniques and genotyping approaches have been used for the detection, diagnosis and characterization of bacteria (Holt, 1994). In each case, the goal has been to distinguish types based on characteristic fingerprints. In general, these approaches are each designed to detect a specific set of variants, but can fail to detect novelty resulting from new introductions or new recombination events. To minimize this problem, Maiden *et al*. (1998) introduced a multilocus sequence typing system (MLST), based on sequencing a short (<1 kb) region of each of several (usually seven) housekeeping genes. The alleles found at each gene are numbered arbitrarily (usually in the order they are found), so that each isolate is represented by the seven numbers corresponding to the alleles at each of the seven genes. Each unique combination of alleles is called a sequence type (ST), and each ST is uniquely numbered. Thus, a given strain can be represented by its ST number, which defines the seven genes specific allele numbers, each of which in turn defines a particular DNA sequence (Maiden, 2006). Subspecies and even host plant related subgroups can be discriminated using this approach (Almeida *et al*., 2008). In addition to being very robust at the subspecies level, any newly discovered *Xf* strain can be assigned to a subspecies, and its phylogenetic placement can be studied in some detail, without the need of working with additional strains for comparisons (Yuan *et al*., 2010). Accordingly, this approach was exploited in the present study, for which the MLST was conducted on sequences of seven housekeeping genes (*leuA, petC, lacF, cysG, holC, nuoL* and *gltT*), together with an additional sequence analysis of different loci responsible for biochemical functions in the *Xf* genome (*RNA Pol sigma-70 factor, hypothetical protein HL, 16S rRNA, rfbD, nuoN,* and *pilU*), all generated from PCR assays conducted on cultured and uncultured *Xf* from affected olives. The results of this study are presented in this paper.

Materials and methods

Sampling olive for *Xylella fastidiosa*

During October–November 2013, a total of 100 samples were collected from two *Xf*-infected olive orchards at the Gallipoli district (southern Italy). Fifty of the samples were from trees showing defoliation, branch dieback and leaf scorch symptoms. Samples consisted of branches and leaves that were placed in individual plastic bags, brought to the laboratory and stored at 4°C to be screened for the presence of *Xf* by polymerase chain reaction (PCR) and double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA).

Isolation of *Xylella fastidiosa*

Attempts were made to isolate the bacterium from 58 infected olive trees (0.1–0.4 g of petioles or midribs) on specific culture media. Pieces of midveins (2 cm long) from plants exhibiting leaf scorch and die-back symptoms were transferred to sterile plastic bags, and were surface disinfected by soaking for 1 min in 70% ethanol and 1 min in 1% sodium hypochlorite followed by three rinses each for 3 min in sterile distilled water. The sample was homogenized aseptically using a semi-automated grinding apparatus (HOMEX; Bioreba) and the sap was poured with a 200 µL sterile pipette tip into a sterile tube. The mix (20 µL drop) was placed on periwinkle wilt gelrite (PWG) or buffered cysteine-yeast extract (BCYE) media (Davis *et al*., 1981a; Wells *et al*., 1981; Hill and Purcell, 1995; Janse *et al*., 2012), incubated at 28°C for 4 weeks and examined with a stereomicroscope at weekly intervals for the presence of *Xylella*-like colonies. Sixty days after plating, the small white colonies were re-streaked three times onto PWG and BYCE media to ensure the purity of the strains. Bacterial cells were afterward observed at 1,000× magnification using light microscopy.

DAS-ELISA

DAS-ELISA was used to check for the presence of *Xf* in collected olive samples and in bacterial cultures using specific antibodies, according to the manufacturer's instructions (Loewe Biochemica GmbH). Briefly, 1 g of leaf midvein tissues from each sample were crushed in the presence of 3 mL of extraction buffer (PBS) using a hammer, at room temperature. In addition, bacterial isolates cultured on PWG and bacterium cells suspended in extraction buffer were also used as antigens. Petiole and midrib tissues of healthy olive trees were processed similarly as negative controls. The results were analyzed by using a plate reader (Bio-Tek KC4, v.3.1). Samples with absorbance values above three times the average absorbance values of the known negative samples were considered positive for X*f*.

DNA extraction and PCR

Genomic DNA was extracted, using the "Qiagen Plant DNAeasy kit" according to the manufacturer's instructions (Qiagen), from 1 g of olive leaf midveins and from 60-d-old bacterial cells of colonies that were scraped from BYCE and PWG agar plates. The concentration of recovered DNA was estimated using a spectrophotometer, and samples were stored at -20°C.

Preliminary molecular identification of parts of the *Xf* genome in olive and bacterial colonies was accomplished using three primer sets targeting the conserved hypothetical protein HL (Francis *et al*., 2006), the RNA polymerase sigma-70 factor (Minesavage *et al*., 1994) and the 16S rDNA genes (Firrao and Bazzi, 1994) (Table 1). In addition, three couples of primers were utilized for PCR genes amplifications (*pilU, nuoN* and *rfbD*), that represented a variety of biochemical functions and were distributed around the *Xf* genome of housekeeping genes (Tables 1 and 3). PCR reactions were carried out using template DNA at 5 ng μL^{-1}, 1.25 U of Go Taq polymerase (Promega), 1× GoTaqFlexi DNA Buffer, 0.2 mM dNTPs, 0.3 µM forward and the reverse primers, all in a final reaction volume of 25 µL. The amplification program consisted of an initial denaturation step at 94°C for 4 min, 40 cycles of 94°C for 45 s, 52–60°C for 30 s (according to each primer, see references in Table 1), and 72°C for 1 min, and a final elongation step of 72°C for 7 min. PCR products were separated by electrophoresis on 1.2% TBE agarose gels run at 7 V cm^{-1} for 1 h, stained with ultraviolet dye (Aurogene Co.) for 10 min, and visualized under UV light.

Multilocus Sequence Typing (MLST)

The genomic DNAs extracted from *Xf* cells of cultured colonies and from infected olive plant material were used as templates to amplify and sequence the seven housekeeping genes (*leuA*, *petC*, *lacF*, *cysG*, *holC*, *nuoL* and *gltT*) described by Scally *et al*. (2005) for a MLST analysis (Table 1). Scally *et al*. (2005) sequenced part of the surface protein gene *pilU* and found that it revealed novel variation not seen in the MLST genes. For this reason, this gene was also included in the present study, thus increasing the diversity of the types of gene monitored in *Xf* from olive; however, this gene is not part of the MLST scheme and was not included in the allelic profiles. For MLST, consensus sequences of all seven amplicons were concatenated into a single sequence (4161 nts) for each strain. Concatenated sequences of the olive strain were aligned with concatenated sequences of selected reference strains available in the GenBank database.

The corresponding sequences for *Xf* subspecies were retrieved from GenBank (accession numbers

Table 1. List of PCR primers used for amplifying different genes of *Xylella fastidiosa* from olive, whose sequences were exploited in the MLST scheme (*HolC*, *nuoL*, *gltT*, *cysG*, *petC*, *leuA* and *lacF*) and in comparative analyses (*RNA pol*, *HL*, *16rRNA*, *pilU*, *rfbD* and *nuoN*) with other *Xf* subspecies.

Gene	Function	Biochemical function	Primer sequences (forward/reverse)	Gene length/ MLST fragment used* (bp)
holC	DNA polymerase III holoenzyme, chi subunit	Replication	5′-GATTTCCAAACCGCGCTTTC-3′ 5′-TCATGTGCAGGCCGCGTCTCT-3′	379/379
nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	Aerobic respiration	5′-CATTATTGCCGGATTGTTAGG-3′ 5′-GCGGGAAACATTACCAAGC-3′	1,821/557
gltT	Glutamate symport protein	Transport of amino acids	5′-TTGGGTGTGGGTACGTTGCTG-3′ 5′-CGCTGCCTCGTAAACCGTTGT-3′	951/654
cysG	Siroheme synthase	Biosynthesis of heme, porphyrin	5′-GGCGGCGGTAAGGTTG-3′ 5′-GCGTATGTCTGTGCGGTGTGC-3′	1,170/600
petC	Ubiquinol cytochrome *c* oxidoreductase, cytochrome *c*1 subunit	Electron transport	5′-CTGCCATTCGTTGAAGTACCT-3′ 5′-CGTCCTCCCAATAAGCCT-3′	533/533
leuA	2-Isopropylmalate synthase	Amino acid biosynthesis	5′-GGGCGTAGACATTATCGAGAC-3′ 5′-GTATCGTTGTGGCGTACACTG-3′	1,218/708
lacF	ABC transporter sugar permease	Transport of carbohydrates	5′-TTGCTGGTCCTGCGGTGTTG-3′ 5′-CCTCGGGTCATCACATAAGGC-3′	730/730
RNA polymerase	RNA polymerase sigma-70 factor	Replication	5′GCGTTAATTTTCGAAGTGATTCGATTGC-3′ 5′-CACCATTCGTATCCCGGTG-3	733
HL	HL protein	Hypothetical protein	5′-AAGGCAATAAACGCGCACTA-3′ 5′-GGTTTTGCTGACTGGCAACA-3′	221
16S rDNA	16S ribosomial DNA	Replication	5′-CAGCACATTGGTAGTAATAC-3′ 5′-ACTAGGTATTAACCAATTGC-3′	404
pilU	Twitching motility protein	Surface structures	5′-CAATGAAGATTCACGGCAATA-3′ 5′-ATAGTTAATGGCTCCGCTATG-3′	915
rfbD	dTDP-4-dehydrorhamnose-3, 5-epimerase	Surface polysaccharides	5′-TTTGGTGATTGAGCCGAGGGT-3′ 5′-CCATAAACGGCCGCTTTC-3′	429
nuoN	NADH-ubiquinone oxidoreductase, NQO14 subunit	Aerobic respiration	5′-GGGTTAAACATTGCCGATCT-3′ 5′-CGGGTTCCAAAGGATTCCTAA-3′	1,398

*According to Scally *et al.*, 2005.

are reported in Figure 2a), and the resulting multiple alignment of concatenated sequences for all subspecies was used as input data to generate the MLST and phylogenetic trees.

Cloning, sequencing and phylogenetic analyses

All PCR amplicons were cloned in StrataClone™ PCR Cloning vector pSC-A (Stratagene), subcloned into *Escherichia coli* DH5α or SoloPACK cells and cus-

tom sequenced (Primm). A consensus sequence was determined for each amplicon based on sequences of four independent clones. Sequence similarities were searched by using BLAST at the National Center for Biotechnology Information website (Altschul *et al*., 1990). Multiple sequence alignments were generated by using the default options of CLUSTALX 1.8 (Thompson *et al*., 1997). For phylogenetic analyses, nucleotide sequences were aligned using MUSCLE (Edgar, 2004), and trees supported by bootstrapping (1,000 times) were generated by the standard "neighbour-joining" method (Perrière and Gouy, 1996), implemented in MEGA 5.05 (Tamura *et al*., 2011). Branches with less than 75% bootstrap support collapsed. Sequences obtained during this study were deposited in the GenBank and their accession numbers are reported in Figure 2 and Table 3.

Results and discussion

For detection of *Xf* in olive samples, results of PCR and ELISA assays were in complete agreement, both showing infection rates of 58% in the 100 olive trees tested, of which 49 showed symptoms of leaf scorch. This result may suggest that the leaf scorch and decline symptoms observed in the fiftieth symptomatic but PCR-negative sample were due to agents other than *Xf*. Moreover, nine samples originating from asymptomatic trees also gave positive PCRs, probably because they were only recently infected by the bacterium. *Xylella fastidiosa* was cultured from one of the 58 PCR-positive samples processed, and the bacterial colonies present on the plate were similar to those reported for *Xf* in literature (Figure 1). Colonies of 0.1–0.3 mm in diameter developed in 2 months after plating, and were observed on BYCE and PWG culture media, but not on PD3 (Davis et al., 1981b; data not presented). ELISA and PCR assays both detected *Xf* from the successful cultured colonies. In particular, 11 *Xf* colonies (chosen randomly) were assayed by PCR, using the 13 couples of primers previously described, and by ELISA, and all showed positive reactions to *Xf*. At the molecular level, all locus sequences generated from PCR amplifications on selected colonies were identical to those of *Xf* present in the respective infected olive plant from which the bacterium was isolated. In addition, sequences were also obtained from other ten *Xf*-infected olive plants. Accordingly, one representative sequence type, denoted "Xf9", was analyzed. Although the *Xf* genome sequences obtained were significantly homogeneous in all analyzed plant sources, the sequences obtained from three different olive trees showed slight nucleotides divergence (0.2%) compared to "Xf9" and accordingly one sequence type, denoted "Xf6", was considered for analysis.

All of the primers used effectively amplified 13 genes in the genome of *Xf* from olive and culture colonies (Table 1). Results of sequence analyses of the *RNA-Pol sigma-70 factor, 16S rDNA, rfbD, nuoN* and *hypothetical protein HL* genes indicated that the olive strain isolated in this study was distinguishable from all reference strains of *Xf* subspecies present in Genbank. In particular, nucleotides Blast analyses of *RNA-Pol sigma-70 factor, 16S rDNA* and *hypothetical protein HL* genes of "Xf6" and "Xf9" showed the greatest identity (99%) with the strain "9a5c" (*Xf* subsp. *pauca*, agent of CVC) (Table 2). Although the sequences of these genes are reported to be highly conserved among *Xf* genomes of different subspecies, here they showed an insignificant variance and gave an insight into the genome sequence of *Xf* from olive, similar to subsp. *pauca* and distinct from all other strains reported in the Genbank. The sequences of *rfbD* and *nuoN* genes were also in line with the three previously mentioned genes for their high identity with those of subsp. *pauca*, strains "9a5c" and "COF0238" (Table 2).

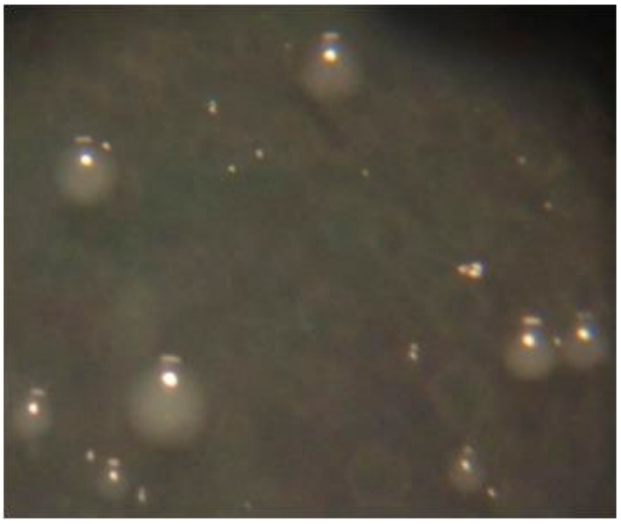

Figure 1. Bacterial colonies of *Xylella fastidiosa* isolated on BYCE medium, 60 d after plating, from infected olive tissue.

Table 2. Maximum nucleotides identity found between *Xylella fastidiosa* from olive (Xf6/Xf9) and those present in the Genbank. Strains of *Xyllela fastidiosa* belonging to the different subspecies *pauca*, *multiplex*, *fastidiosa, sandyi* and those isolated from olive (Italy), are represented by $^{p, m, f, s, o}$, respectively. Accession numbers respectively for Xf6 and Xf9 are the following: *pilU* (HG939501, LM999931), *RNA pol* (HG939492, LM999922), *16S rDNA* (HG939494, LM999924), *rfbD* (HG939496, LM999926), *nuoN* (HG939498, LM999928) and *HL* (HG939493, LM999923). Accession numbers relative to strains are reported in order.

Genes Xf6/Xf9	Strain	Identity (%)	Accession numbers
pilU	9a5c^{p}, CVC0018^{p}	99	AE003849, FJ610227
	Ann1^{s}, RIV5^{m}, M12^{m}, MUL0034^{m}, GB514^{f}, Temecula1^{f}, OAK2^{m}, ALS2^{m}, Dixon^{m}, RIV^{s}, TR2^{s}	98	CP006696, JX679707, CP00941, CP006740, CP002165, AE009442, AY876845, AY876840, AY876838, AY876834, AY876836
	M23^{f}, 95-2^{f}, I03^{f}	97	CP001011, AY876829, AY876827
RNA pol	OLG2^{o}, PW1^{o}, ALM1^{o}, 9a5c	99	KJ406214, KJ406260, KJ406262, AE003849
	GB514, M23, Temecula1	98	CP002165, CP001011, AE009442
	M12^{m}	97	CP000941
16S rDNA	OLG2, M12, Ann1, 9a5c, GB514, M23, Temecula1	99	KJ406215, CP000941, CP006696, AE003849, CP002165, CP001011, AE009442
rfbD	Ann1, M12, COF0238^{p}	98	CP006696, CP00941, JQ290506
	GB514, M23, Temecula1, 9a5c	97	CP002165, CP001011, AE009442, AE003849
nuoN	9a5c	99	AE003849
	Dixon, M12	97	AY876813, CP000941
	Ann1, GB514, M23, Temecula1	96	CP006696, CP002165, CP001011, AE009442
HL	OLG2, 9a5c	99	KJ406211, AE003849
	Ann1, GB514, M23, M12, Temecula1	96	CP006696, CP002165, CP001011, CP000941, AE009442

Analyzing the gene coding the cell-surface protein *pilU*, which is reported to enclose novel variation not seen in those of MLST, corresponding sequences of "Xf6" and "Xf9" also showed the highest identity (99%) with two *Xf* strains of subsp. *pauca*. ("9a5c" and "CVC0018").

The total length of the MLST of all seven loci sequenced was 4,161 bp. The allelic profiles of each sequence type (ST) and the ST of each isolate are shown in Table 3. The two isolates sequenced in this study (Xf6 and Xf9) formed a genetically uniform clonal complex made up of a single ST (ST53) together with *Xf* isolates from other hosts (almond, oleander and periwinkle) reported to be infected with the same strain in southern Italy and whose sequences are deposited in Genbank. The MLST analysis showed that the *Xf* isolates from southern Italy were different from all the other *Xf* subspecies already reported in the literature. They shared only three allelic profiles (*LeuA*, *petC* and *holC*) with subsp. *pauca* (9a5c and CVC0018), whereas the four remaining loci analyzed (*lacF*, *cysG*, *nuoL* and *gltT*) were different (Table 3).

Analyzing the MLST data of isolates 9a5c and CVC0018 (subsp. *pauca*), which shared certain allelic profiles (Xf6, Xf9, OLG2, KM13, PW1 and ALM1) with those from Apulia, there was no indication that any of the gene regions targeted was subject to positive selection. The overall ratio of non-synonymous to synonymous substitution (dn/ds) was 0.017, well below the positive selection threshold of 1.

The two phylogenetic analyses here adopted were based on the *Xf* MLST concatameric sequences,

Table 3. Allelic profiles for sequence types (STs) found in the multilocus sequence typing (MLST) of different *Xylella fastidiosa* isolates belonging to different subspecies. The allele numbers are arbitrary (similar numbers do not represent sequence similarity). ST defines the sequence type designation for each strain. Numbers in bold represent the same locus type between CoDiRO and *pauca* strains, whereas those underlined are different.

Strain/ Genotype	Host species	MLST loci								
		leuA	*petC*	*lacF*	*cysG*	*holC*	*nuoL*	*gltT*	Subspecies/ Strain	ST
Xf6	Olive	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
Xf9	Olive	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
OLG2	Olive	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
KM13	Olive	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
OLDR-1	Oleander	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
PW1	Periwinkle	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
ALM1	Almond	**7**	**6**	**16**	**24**	**10**	**16**	**14**	*pauca/ CoDiRO*	**53**
9a5c	Citrus	**7**	**6**	*7*	*9*	**10**	*7*	*8*	*pauca*	*13*
CVC0018	Citrus	**7**	**6**	*7*	*9*	**10**	*7*	*8*	*pauca*	*13*
Dixon	Almond	3	3	3	3	3	3	3	*multiplex*	6
M12	Almond	3	3	3	3	3	3	3	*multiplex*	7
GB514	Grapevine	1	1	1	1	1	1	1	*fastidiosa*	1
M23	Citrus	1	1	1	1	1	1	1	*fastidiosa*	1
Temecula1	Grapevine	1	1	1	1	1	1	1	*fastidiosa*	1
Ann1	Grapevine	2	2	2	2	2	2	2	*sandyi*	5

considered as an efficient template for strain delineation, and on the *pilU* locus which is generally used to increase the power of genetic differentiation within the subspecies. The phylogentic tree generated from alignment of the concatameric sequences confirms the results obtained from *pilU, RNA-Pol sigma-70 factor, 16S rDNA, rfbD, nuoN* and *HL*-based analyses. This further indicates that "Xf6" and "Xf9" isolates and those reported to infect other hosts in southern Italy are related to each other, and are closer to, but distinct from, *Xf* subsp. *pauca* (9a5C, CVC0018), and also distinct from other reported *Xf* isolates (Figure 2a and b). The *Xf* from the olive samples in southern Italy thus clustered into a unique clade distinct from those containing other recognized subspecies.

Based on the type of molecular analysis here conducted (MLST), which is the minimum standard needed to characterize a new bacterial strain, it is now legitimate to definitely attribute to the Apulian strain of *Xylella fastidiosa* the name of "CoDiRO" within the subspecies *pauca.* as previously inferred by Cariddi *et al.* (2014) even though based on the sequence of the single gene DNA *gyrase* subunit B (gyrB).

The genetic vicinity of "CoDiRO" to the *pauca* lineage raises concerns for growers in southern Italy. This is because this strain is potentially pathogenic to hosts other than olive, in particular to *Citrus* spp. that are heavily affected by *Xf*. subsp. *pauca* (the agent of CVC disease). To date, however, the results of preliminary investigations exclude the ability of the Apulian *Xf* strain to infect *Citrus* spp. (D. Boscia, personal communication).

Whether this *Xf* strain is pathogenic to olive and to other species in southern Italy has yet to be determined through pathogenicity assays. Neverthe-

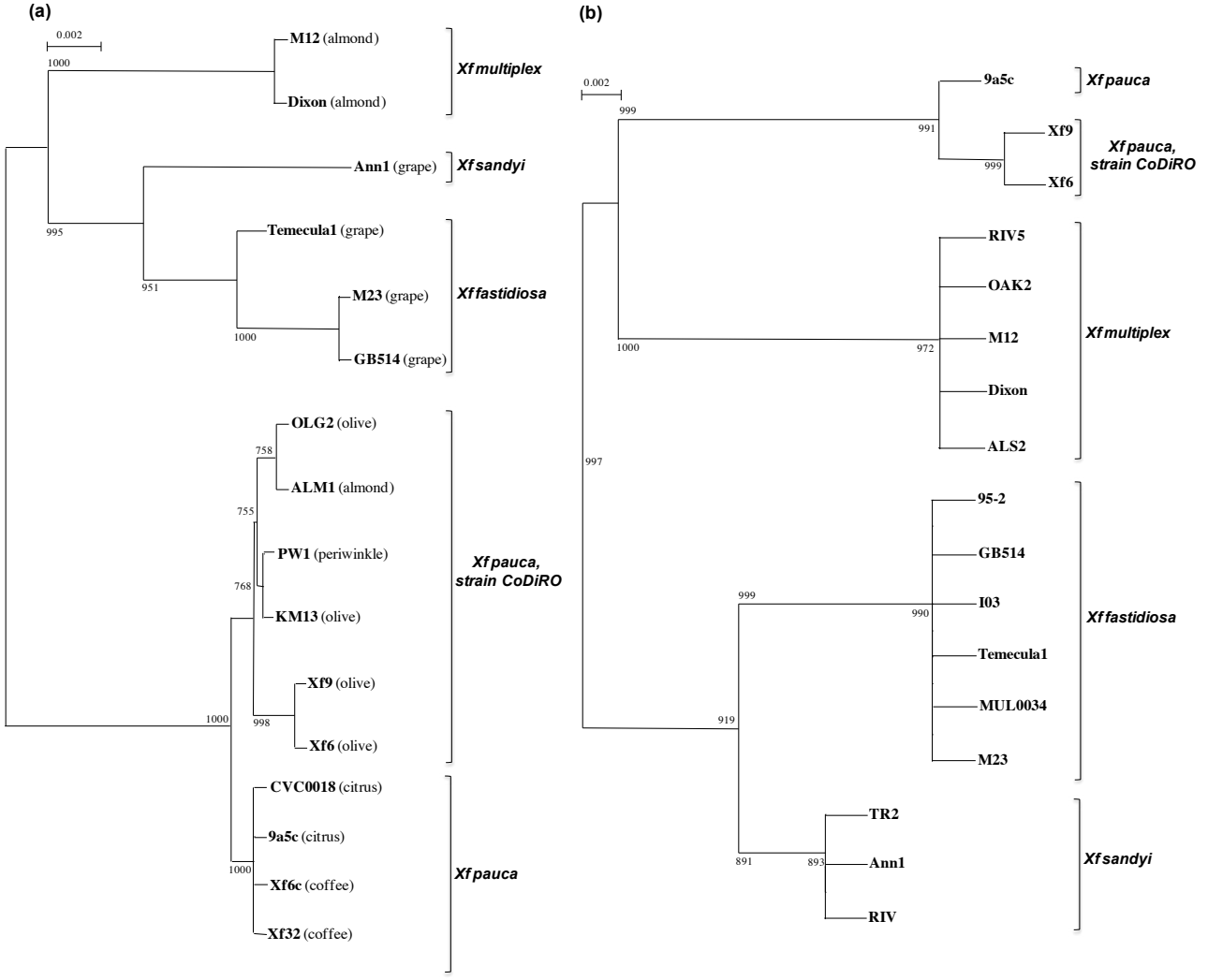

Figure 2. (a) Concatameric phylogram based on sequences of seven different loci (*leuA*, *petC*, *lacF*, *cysG*, *holC*, *nuoL*, *gltT)* used in MLST that were linked in order for each *Xylella fastidiosa* isolate. The maximum-likelihood method using MEGA5.0 was utilized to construct both phylogenetic trees, that were bootstrapped 100 times (values >75% are shown). Bootstrapping consensus values are shown on trees. Isolates included olive isolates as well as strains of *X. fastidiosa* belonging to different subspecies: M12 (GenBank accession CP000941), Dixon (AAAL02000003), Ann1 (CO006696), Temecula1 (AE009442,AY876648), M23 (CP001011), GB514 (CP002165), OLG2 (KJ406216, KJ406222, KJ406228, KJ406252, KJ406234, KJ406240, KJ406246), ALM1 (KJ406220, KJ406226, KJ406232, KJ406256, KJ406238, KJ406244, KJ406250), PW1 (KJ406221, KJ406227, KJ406233, KJ406257, KJ406239, KJ406245, KJ406251), KM13 (KJ406218, KJ406224, KJ406230, KJ406254, KJ406236, KJ406242, KJ406248), Xf6 (HG939499, HG939497, HG939503, LM999935, HG939495, HG939502, HG939500), Xf9 (LM999929, LM999927, LM999933, LM999934, LM999925, LM999932, LM999930), Xf32 (AWYH00000000), Xf6c (NZ_AXBS00000000), CVC0018 (FJ610159, FJ610168, FJ610176, FJ610185, FJ610197, FJ610207, FJ610217) and 9a5c (AE003849). (b) Single-locus phylogenetic tree based on sequences of *pilU*. Accession numbers of isolates used in the tree are reported in Table 3.

less, the laboratory screening (PCR and ELISA) conducted on 100 samples from trees showing defoliation and shoot dieback provide evidence of the strict association of this bacterium with the quick decline symptoms, since 49 out of 50 symptomatic olive trees were positive for the presence of this bacterium.

Moreover, the leaf scorch symptoms observed were similar to those described for *Xf* infections on olive in California, USA (Krugner *et al*., 2014). Whether the OQDS is a result of a co-infection by other pests (fungi and\or insects) present on olive in Apulia region (Nigro *et al*., 2014) or related solely to the bacterial strain is yet unknown.

The difficulty to isolate *Xf* on culture media from a large number of symptomatic olive plants is probably due to the fastidious nature of the bacterium, which is influenced by biotic and abiotic factors or may be at low concentrations in affected plants. The *Xf* isolated on culture media is currently being used in inoculations of olive and other plant hosts, for fulfillment of Koch's Postulates, and to study the pathogenicity of this particular bacterium strain.

Literature cited

Almeida R.P.P., F.E. Nascimento, J. Chau, S.S. Prado, C.W. Tsai, S.A. Lopes and J.R.S. Lopes, 2008. Genetic structure and biology of *Xylella fastidiosa* strains causing disease in citrus and coffee in Brazil. *Applied and Environmental Microbiology* 74, 3690–3701.

Altschul S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Barnard E.L., E.C. Ash, D.L. Hopkins and R.J. McGovern, 1998. Distribution of *Xylella fastidiosa* in oaks in Florida and its association with growth decline in *Quercus laevis*. *Plant Disease* 82, 569–572.

Buzombo P., J. Jaimes, V. Lam, K. Cantrell, M. Harkness, D. Mc-Cullough and L. Morano, 2006. An American hybrid vineyard in the Texas Gulf Coast: Analysis within a Pierce's disease hotzone. *American Journal of Enology and Viticulture* 57, 347–355.

Cariddi C., M. Saponari, D. Boscia, A. De Stradis, G. Loconsole, F. Nigro, F. Porcelli, O. Potere and G.P. Martelli, 2014. Isolation of a *Xylella fastidiosa* strain infecting olive and oleander in Apulia, Italy. *Journal of Plant Pathology* 96(3), 1–5.

Chang C.J., M. Garnier, L. Zreik, V. Rossetti and J.M. Bove, 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology* 27, 137–142.

Davis M.J., A.H. Purcell and S.V. Thomson, 1978. Pierce's disease of grapevines isolation of the casual bacterium. *Science* 199, 75–77.

Davis M.J., W.J. French and N.W. Schaad, 1981a. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Current Microbiology* 6, 309–314.

Davis M.J., Whitcomb R.F., Gillaspie A.G.J., 1981b Fastidious bacteria of plant vascular tissue and invertebrates (including so called rickettsia-like bacteria). In: The prokaryotes: a handbook on habits, isolation, and identification of bacteria (Starr M.P., Stolp H., Truper Hg., Balows A., Schlegel Hg., ed.), Berlin, Heidelberg: Springer -Verlag, pp. 2172–2188.

Edgar R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32, 1792–1797.

Elbeaino T., T. Yaseen, F. Valentini, I.E. Ben Moussa, V. Mazzoni and A.M. D'onghia, 2014. Identification of three potential insect vectors of *Xylella fastidiosa* in Southern Italy. *Phytopathologia Mediterranea* 53(1), 126–130.

Firrao G. and C. Bazzi, 1994. Specific identification of *Xylella fastidiosa* using the polymerase chain reaction. *Phytopathologia Mediterranea* 33, 90–92.

Francis M., H. Lin, J. Cabrera-La Rosa, H. Doddapaneni and E.L. Civerolo, 2006. Genome-based PCR Primers for Specific and Sensitive Detection and Quantification of *Xylella fastidiosa*. *European Journal of Plant Pathology* 115, 203–213.

Janse J.D, F. Valentini, A.H. Purcell, and R.P.P. Almeida, 2012. Detection and identification methods and new tests as used and developed in the framework of COST873 for bacteria pathogenic to stone fruits and nuts-*Xylella fastidiosa. Journal of Plant Pathology* 94(1, Suppl.), S147–S154.

Hernandez-Martinez R., T.R. Pinckard, H.S. Costa, D.A. Cooksey and F.P. Wong, 2006. Discovery and characterization of *Xylella fastidiosa* strains in southern California causing mulberry leaf scorch. *Plant Disease* 90, 1143–1149.

Hill B.L. and A.P. Purcell, 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathology* 85, 1368–1372.

Holt J.G., 1994. Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Wilkins, Baltimore, MD.

Krugner R., M.S. Sisterson, J. Chen, D.C. Stenger and M.W. Johnson, 2014. Evaluation of olive as a host of *Xylella fastidiosa* and associated sharpshooter vectors. *Plant Disease* 98, 1186–1193.

Li W.B., W.D. Pria, C. Teixeira, V.S. Miranda, A.J. Ayres, C.F. Franco, M.G. Costa, C.X. He, P.I. Costa and J.S. Hartung, 2001. Coffee leaf scorch caused by a strain of *Xylella fastidiosa* from citrus. *Plant Disease* 85, 501–505.

Maiden M.C.J., 2006. Multilocus sequence typing of bacteria. *Annual Review of Microbiology* 60, 561–588.

Maiden M.C.J., J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, I.M. Feavers, M. Achtman and B.G. Spratt, 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Science* 95, 3140–3145.

Minesavage G.V., C.M. Thompson, D.L. Hopkins, M.V.B.C. Leite and R.E. Stall, 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology* 84, 456–461.

Mircetich S.M., S.K. Lowe, W.J. Moller and G. Nyland, 1976. Etiology of almond leaf scorch disease and transmission of the casual agent. *Phytopathology* 66, 17–24.

Nigro F., D. Boscia, I. Antelmi and A. Ippolito, 2013. Fungal species associated with a severe decline of olive in southern Italy. *Journal of plant pathology* 95, 668.

Nunney L., X. Yuan, R. Bromley, J. Hartung, M. Montero-Astua, L. Moreira, B. Ortiz and R. Stouthamer, 2010. Population genomic analysis of a bacterial plant pathogen: Novel insight into the origin of Pierce's disease of grapevine in the US. *PloS ONE* 5(11), e15488.

Nunney L., Yuan X., Bromley R.E. and Stouthamerb R., 2012. Detecting genetic introgression: high levels of intersubspecific recombination found in *Xylella fastidiosa* in Brazil. *Applied and Environmental Microbiology* 78, 4702.

Perrière G. and M. Gouy, 1996. WWW-Query: An on-line retrieval system for biological sequence banks. *Biochimie* 78, 364–369.

Purcell A.H., S.R. Saunders, M. Hendson, M.E. Grebus and M.J. Henry, 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathology* 89, 53–58.

Raju B.C., J.M. Wells, G. Nyland, R.H. Berlansky and S.K. Lowe, 1982. Plum leaf scald isolation and culture and pathogenicity of the causal agent. *Phytopathology* 72, 1460–1466.

Sanderlin R.S. and K.I. Heyderich-Alger, 2000. Evidence that *Xylella fastidiosa* can cause leaf scorch disease of pecan. *Plant Disease* 84, 1282–1286.

Saponari M., D. Boscia, F. Nigro and G.P. Martelli, 2013. Identification of DNA sequences related to Xylella fastidiosa in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). *Journal of Plant Pathology* 95, 659–668.

Saponari M., G. Loconsole., D. Cornara, R.K. Yokomi, A. De Stradis, D. Boscia, D. Bosco, G.P. Martelli, R. Krugner and F. Porcelli, 2014. Infectivity and transmission of *Xylella fastidiosa* by Philaenus spumarius (Hemiptera: Aphrophoridae) in Apulia, Italy. *Journal of Economic Entomology* 107(4), 1316–1319.

Scally M., E.L., Schuenzel, R. Stouthamer and L., Nunney, 2005. A multilocus sequence type system for the plant pathogen *Xylella fastidiosa*, and the relative contribution of recombination versus point mutation to clonal diversity. *Applied and Environmental Microbiology* 71, 8491–8499.

Schaad N.W., E. Postnikova, G. Lacy, M. Fatmi and C.J. Chang, 2004. *Xylella fastidiosa* subspecies: X. fastidiosa subsp. piercei subsp. nov., X. fastidiosa subsp. multiplex subsp. nov., X. fastidiosa subsp. pauca subsp. nov. Syst. *Applied and Environmental Microbiology* 27, 290–300.

Schuenzel E., M. Scally, R. Stouthamer and L. Nunney, 2005. A multigene phylogenetic study of clonal diversity and divergence in North American strains of the plant pathogen Xylella fastidiosa. *Applied and Environmental Microbiology*, 71, 3832-3839.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology Evolution* 28, 2731–2739.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24, 4876–4882.

Wells J.M., B.C. Raju, G. Nyland and S.K. Lowe, 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. *Applied and Environmental Microbiology* 42, 357–363.

Wells J.M., B.C. Raju and G. Nyland, 1983. Isolation culture and pathogenicity of the bacterium causing phony disease of peach *Prunus persica*. *Phytopathology* 73, 859–862.

Wells J.M., B.C. Raju, H.Y. Hung, W.G. Weisburg, L. Mandelco-Paul and D.J. Brenner, 1987. *Xylella fastidiosa*: Gram-negative, xylem-limited, fastidious plant bacteria related to Xanthomonas. *International Journal of Systemic Bacteriology* 37, 136–143.

Yuan X., L. Morano, R. Bromley, S. Spring-Pearson, R. Stouthamer and L. Nunney, 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. *Phytopathology* 100, 601–611.

Accepted for publication: October 31, 2014
Published online: December 22, 2014