Nanopore sequencing for the detection and identification of *Xylella fastidiosa* subspecies and sequence types from naturally infected plant material

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Abstract

Xylella fastidiosa (Xf) is a gram-negative bacterial plant pathogen that can infect over 500 plant species. While it is endemic in America, *X. fastidiosa* subsp. *pauca* was reported for the first time in Europe in 2013 on olive trees in southern Italy. The availability of fast, sensitive, and reliable diagnostic tools is indispensable for managing current and future outbreaks of Xf. In this paper, we use the Oxford Nanopore Technologies (ONT) MinION platform for detecting and identifying Xf at species, subspecies, and sequence type (ST) level. Two workflows were developed: the first one provided a "shotgun" strategy, that is, exploring the possibility of detecting Xf within DNA extracted from plant samples. This allowed detection of Xf by direct DNA sequencing and identifying the subspecies only in samples with high bacterial levels. Nanopore amplicon sequencing was pursued as a second workflow. This consists of PCR amplification of a set of seven multilocus sequence typing (MLST) fragments, officially adopted for identifying Xf at type strain level, followed by Nanopore-sequencing of the amplicons and an ad hoc pipeline to generate MLST consensus calls. This combined approach, which takes only a few hours, allowed the detection and identification of Xf at ST level in plant material with low bacterial infection.

KEYWORDS

MinION, pathogen detection, *Xylella fastidiosa*

1 | INTRODUCTION

Xylella fastidiosa (Xf), a gram-negative phytopathogenic bacterium (Wells et al., 1987), has a very broad host range, causing different diseases in important crops (Hopkins, 1989) and in many urban shade trees (Sherald & Kostka, 1992). Xf symptoms may not be

evident and many hosts can carry symptomless infections, making this pathogen difficult to manage. The pathogen is transmitted by xylem sap-feeding insects and colonizes host xylem vessels, causing typical leaf scorching. Xf is an endemic pathogen in America and was only recently identified in southern Italy on olive trees (Saponari et al., 2013), in Corsica, and in the south-east Mediterranean coast

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of France on several hosts (Denancé et al., 2019). Subsequently, outbreaks of Xf were reported in Spain (Mallorca and Andalusia) in different plant species (Landa, 2017; Olmo et al., 2017) and in Portugal (EPPO, 2019a). Recently, a new occurrence was also reported in Tuscany, Italy (Marchi et al., 2018). Different Xf subspecies and sequence types (ST) were identified in Europe in association with the above-mentioned outbreaks. Based on DNA–DNA hybridization and recent comparative genomic analyses, three subspecies of Xf are recognized, namely *fastidiosa*, *pauca*, and *multiplex* (Denancé et al., 2019; Marcelletti & Scortichini, 2016; Schaad et al., 2004). However, the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) considers only the subsp. *fastidiosa* and subsp. *multiplex* as valid names, whereas other authors classify five subspecies (*fastidiosa*: Xff; *pauca*: Xfp; *multiplex*: Xfm; *sandyi*: Xfs; *morus*: Xfmo) (Bull et al., 2014; Nunney et al., 2012). Moreover, Xf subspecies include different sequence types (STs) that are determined by multilocus sequence typing (MLST) analysis based on the sequencing of seven housekeeping genes (EPPO, 2019b; Nunney et al., 2012; Yuan et al., 2010). MLST analysis is recommended for unambiguous identification of subspecies and ST in the case of a new outbreak or new plant hosts (EPPO, 2019b). Isolation of Xf is tedious, but the MLST analysis can be performed directly on DNA isolated from the host plant (EPPO, 2019b). However, a low concentration of the bacteria, and contaminants derived from the plant material, makes the MLST amplification, and thus subspecies and ST determination, extremely challenging (EPPO, 2019b).

Currently, next-generation sequencing (NGS) platforms represent high-throughput technologies that allow large data sets of genetic information to be obtained. In the biomedical field, sequencing technologies are rapidly becoming adopted for bacterial outbreak investigations (Faria et al., 2017) and for rapid clinical diagnostics (Votintseva et al., 2017). A combination of whole transcriptome amplification and Nanopore sequencing has been used to detect '*Candidatus Liberibacter asiaticus*' and plum pox virus in plants and in insect vectors (Badial et al., 2018). The reliability of direct Nanopore sequencing for the diagnosis of several plant pathogens was demonstrated in artificially infected plants showing symptoms (Chalupowicz et al., 2019), and a rapid MLST determination method was described for ST estimation of *Klebsiella pneumoniae* isolates using Nanopore sequencing (Page & Keane, 2018).

Diagnostic procedures under global trading have to be fast, reliable, and quantitative. Furthermore, the movement of pathogens and pests is becoming more prevalent thanks to globalization of commerce and global warming. The level of precision required to determine the species and to prevent its entrance into a pest-free area is currently at a maximum level. For bacteria at least, the identification should go down to subspecies or strain level, allowing effective control strategies to be designed promptly. In our study we propose a strategy that exploits Oxford Nanopore Technologies (ONT) to diagnose emerging and invasive pathogens such as Xf. For this, we investigated two strategies: one relies on a shotgun approach, that is, direct sequencing of plant extracts, and the other on amplicon

sequencing. The latter was the most reliable and robust strategy for subspecies and ST determination.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

Four data sets were prepared as follows. The first data set (Set 1) consisted of 21 DNA samples extracted from naturally infected olive plants (*Olea europaea*) collected in the Apulia region of southern Italy. Leaf samples collected as described by Scortichini et al. (2018) were crushed in a plastic bag (Bioreba) containing 5 ml of Food Lysis Buffer from the DNeasy mericon Food Kit (50) (Qiagen) and DNA extraction was performed following the PM 7/24 (4) (EPPO, 2019b). Set 2 consisted of naturally infected samples of *Spartium junceum*, *Polygala myrtifolia*, *Rosmarinus officinalis*, *Rhamnus alaternus*, *Prunus amygdalus*, *Cistus* spp., and *Lavandula* spp., collected in Tuscany. Set 3 consisted of DNA from healthy olive trees collected in Latium, Italy, spiked with a known concentration of DNA from Xfp strain CFBP 8402. A pure culture of Xfp strain CFBP 8402, isolated from olive in Apulia region, was grown for 7–10 days at 28 °C on buffered charcoal yeast extract (BCYE) agar. The culture was then scraped from the plate and resuspended in 100 µl of phosphate buffer (PB) and grown in 10 ml of PD2 broth at 28 °C, 170 rpm for 7–10 days up to a suspension of $OD_{600} = 0.8$. The bacterial DNA was extracted from 700 µl of culture medium using the Gentra Puregene Yeast/Bact Kit (Qiagen). DNA from healthy olive leaves was extracted as reported below. The sample was collected from an olive plant located in a pest-free area in Rome and previously screened for the absence of Xf by real-time PCR (Harper et al., 2010). DNA concentration was quantified with a DS-11 FX+spectrophotometer (DENOVIX). DNA (about 40 ng/µl) of healthy olive samples was amended with 100, 10, 8, or 4 fg of Xfp DNA per PCR, each in three independent replicates. These Xf DNA concentrations were chosen as they corresponded to values close to the detection limit of real-time PCR, estimated according to Modesti et al. (2017), and equivalent to 10 fg/PCR for the real-time PCR of both Harper et al. (2010) and Francis et al. (2006).

The last data set, Set 4, consisted of one healthy sample of different plant species collected in an Xf-free area from Latium region. DNA extraction of plant material from Sets 2, 3, and 4 were performed with the CTAB-based method as reported in PM7/24 (4) (EPPO, 2019b).

2.2 | Real-time PCR and MLST

TaqMan real-time PCR (Harper et al., 2010) was performed using 1 µl of total DNA in a final volume of 11 µl (Loreti et al., 2017), and TaqMan real-time PCR (Francis et al., 2006) using 1 µl of total DNA in a final volume of 10 µl. The TaqMan Fast Universal Master Mix, no AmpErase UNG (Applied Biosystems), and TaqMan Universal PCR Master Mix (ThermoFisher Scientific, 2×) were used for real-time

PCR according to Harper et al. (2010) and Francis et al. (2006), respectively. These two real-time PCRs are currently used for the detection of Xf and both are considered reliable methods based on a recent EPPO validation report (Saponari & Loconsole, 2018). All samples were also tested by real-time PCR as described by Li et al. (2013), following PM7/24 (4) (EPPO, 2019b) to confirm the bacterial infection. MLST analysis was performed on *Cystus*, *Lavandula*, and *Rosmarinus* as previously described (EPPO, 2019b; Yuan et al., 2010) and following PM7/24 (4) (EPPO, 2019b). For Nanopore amplicon sequencing, the amplification of housekeeping genes was modified by increasing the number of PCR cycles from 35 to 40 and by using Immolase DNA polymerase (Bioline).

2.3 | Data sets used for Nanopore sequencing

Direct Nanopore DNA sequencing was performed on Sets 1, 2, and 3 (Tables 1–3). Set 3 was used to test the direct DNA Nanopore sequencing near the limit of detection (LoD) of the real-time PCR (Table 3). DNA samples from Sets 2, 3, and 4 were used for Nanopore amplicon sequencing of the two genes *cysG* and *malF*. Three out of

seven samples from Set 2 (i.e., *Cystus*, *Lavandula*, and *Rosmarinus*) were assessed with Nanopore amplicon sequencing for all seven housekeeping genes.

2.4 | Nanopore sequencing

Nanopore sequencing libraries were prepared according to the manufacturer's instructions for the kit SQK-RBK004 for both direct DNA and amplicon sequencing and sequenced on a R9.4.1 flowcell on a Mk1B device (Oxford Nanopore Technologies). In brief, about 400 ng DNA was purified using AMPure beads, ligated to the indexing adapter, combined in one sample, and subsequently ligated to the RAP adapter prior to sequencing. For amplicon sequencing, PCR amplicons of each sample were pooled and purified using Isolate II PCR and gel Kit (Bioline) and about 100 ng DNA was used for library preparation. DNA samples were run on the Nanopore flowcell v. 9.4.1 until pore life ended, while amplicon sequencing runs were performed for shorter time spans (c.5 hr). After sequencing, Deepbinner (v. 0.2.0) (Wick et al., 2018) was used to demultiplex the samples using default parameters. Subsequently, basecalling and

TABLE 1 Direct Nanopore sequencing of *Xylella fastidiosa* (Xf) DNA in naturally infected olive samples collected in the Apulia region in Italy (data Set 1)

Sample	C _t ^a	Mb	No. of reads	No. of reads mapped (%) ^b	No. of reads mapping to bacteria (%) ^c	No. of reads mapping to Xf (%) ^d
Olive-1	21.28	140.47	46,444	41,478 (89.3)	755 (1.8)	111 (14.7)
Olive-2	21.44	227.67	80,039	70,081 (87.6)	1,236 (1.8)	188 (15.2)
Olive-3	21.92	113.07	34,789	31,012 (89.1)	556 (1.8)	49 (8.8)
Olive-4	25.7	100.87	44,308	39,342 (88.8)	539 (1.4)	5 (0.9)
Olive-5	25.5	62.23	33,632	28,633 (85.1)	367 (1.3)	3 (0.8)
Olive-6	25.3	68.11	33,152	28,773 (86.8)	384 (1.3)	6 (1.6)
Olive-7	27.85	107.05	39,608	34,853 (88.0)	537 (1.5)	9 (1.7)
Olive-8	27.24	95.05	33,240	29,871 (89.9)	525 (1.8)	2 (0.4)
Olive-9	27.74	102.02	39,916	35,393 (88.7)	580 (1.6)	12 (2.1)
Olive-10	35.3	43.94	17,468	15,383 (88.1)	213 (1.4)	0 (0.0)
Olive-11	36.17	160.30	52,157	46,588 (89.3)	827 (1.8)	0 (0.0)
Olive-12	35.06	43.47	18,259	15,689 (85.9)	249 (1.6)	0 (0.0)
Olive-13	28	69.15	30,135	25,379 (84.2)	325 (1.3)	4 (1.2)
Olive-14	26	111.75	42,884	37,284 (86.9)	538 (1.4)	61 (11.3)
Olive-15	34.5	87.21	34,260	29,717 (86.7)	448 (1.5)	1 (0.2)
Olive-16	33.75	94.79	42,478	36,246 (85.3)	480 (1.3)	0 (0.0)
Olive-17	31.6	31.19	10,868	9,586 (88.2)	131 (1.4)	0 (0.0)
Olive-18	30.1	101.70	43,913	37,632 (85.7)	475 (1.3)	3 (0.6)
Olive-19	25.3	81.98	28,591	24,781 (86.7)	402 (1.6)	22 (5.5)
Olive-20	36	128.11	37,611	33,844 (90.0)	543 (1.6)	0 (0.0)
Olive-21	29	106.21	35,496	31,236 (88.0)	540 (1.7)	4 (0.7)

^aCycle threshold obtained by Harper et al. (2010), TaqMan version.

^bPercentage of reads mapped to bacterial species and *Olea europaea* over the number of total reads sequenced.

^cPercentage of reads mapped to bacterial species from the total mapped.

^dPercentage of reads mapped to Xf over the number of reads mapped to bacterial species.

TABLE 2 Direct Nanopore sequencing of *Xylella fastidiosa* (Xf) DNA in naturally infected plant samples collected in the Tuscany region in Italy (data Set 2)

Sample	C _t ^a	Mb	No. of reads	No. of reads mapped (%) ^b	No. of reads mapping to bacteria (%) ^c	No. of reads mapping to Xf (%) ^d
<i>Prunus amygdalus</i>	27.35	122.78	114,327	11,508 (10.1)	7,621 (66.2)	22 (0.3)
<i>Rhamnus alaternus</i>	NA	73.45	62,664	4,335 (6.9)	2,737 (63.1)	10 (0.4)
<i>Cystus</i> sp.	25.78	606.32	199,423	5,156 (2.6)	3,522 (68.3)	230 (6.5)
<i>Rosmarinus officinalis</i>	33.00	247.79	182,831	9,657 (5.3)	4,662 (48.3)	2 (0.1)
<i>Lavandula</i> sp.	28.75	438.39	211,320	25,197 (11.9)	10,231 (40.6)	86 (0.8)
<i>Polygala myrtifolia</i>	25.10	184.91	87,762	11,447 (13.0)	6,157 (53.8)	104 (1.7)
<i>Spartium juceum</i>	18.61	286.19	155,150	10,919 (7.0)	6,650 (60.9)	1,699 (25.5)

^aCycle threshold obtained by Francis et al. (2006), TaqMan version.^bPercentage of reads mapped to bacterial species and *Olea europaea* over the number of total reads sequenced.^cPercentage of reads mapped to bacterial species from the total mapped.^dPercentage of reads mapped to Xf genomes over the number of reads mapped to bacterial species.**TABLE 3** Direct Nanopore sequencing of DNA from healthy olive samples spiked with a known concentration of DNA from *Xylella fastidiosa* (Xf) subsp. *pauca* CFBP 8402 (data Set 3)

Sample	C _t ^a	Xf gDNA (fg/PCR)	Xf gDNA (fg/Nanopore reaction) ^b	Mb	No. of reads	No. of reads mapped (%) ^c	No. of reads mapping to bacteria (%) ^d	No. of reads mapping to Xf (%) ^e
LOD-1	31.5–32.3	100	700	33.63	21,218	16,334 (77.0)	615 (3.8)	2 (0.3)
LOD-2	31.0–31.3	100	700	7.97	6,656	3,853 (57.9)	105 (2.7)	0 (0.0)
LOD-3	31.2–30.7	100	700	17.51	16,237	8,820 (54.3)	308 (3.5)	1 (0.3)
LOD-4	34.5–34.4	10	70	26.35	22,475	15,047 (66.9)	542 (3.6)	0 (0.0)
LOD-5	33.1–33.6	10	70	26.31	21,669	14,915 (68.8)	486 (3.3)	1 (0.2)
LOD-6	33.2–34.0	10	70	31.99	24,385	16,836 (69.0)	597 (3.5)	0 (0.0)
LOD-7	37.3–44.0	8	56	27.69	21,949	14,693 (66.9)	475 (3.2)	0 (0.0)
LOD-8	35.0–35.5	8	56	35.64	34,572	23,594 (68.2)	725 (3.1)	0 (0.0)
LOD-9	40.0–42.0	8	56	22.15	29,800	18,724 (62.8)	455 (2.4)	0 (0.0)
LOD-10	37.4–NA	4	28	39.51	38,724	26,887 (69.4)	740 (2.8)	1 (0.1)
LOD-11	36.0–NA	4	28	45.19	45,824	30,053 (65.6)	833 (2.8)	0 (0.0)
LOD-12	41.0–NA	4	28	10.07	21,082	10,627 (50.4)	272 (2.6)	0 (0.0)

^aCycle threshold obtained by Francis et al. (2006), TaqMan version. The value reports the C_t interval for the three technical replicates used for each sample. NA, low concentrate samples gave technical replicates with no C_t value.^bTotal amount of Xf subsp. *pauca* gDNA added to *Olea europaea* gDNA and used for Nanopore sequencing.^cPercentage of reads mapped to bacterial species and *O. europaea* over the number of total reads sequenced.^dPercentage of reads mapped to bacterial species from the total mapped.^ePercentage of reads mapped to Xf over the number of reads mapped to bacterial species.

a new run of demultiplexing was performed using Guppy (v. 2.3.1; default parameters) (Wick et al., 2019). Deepbiner and guppy base-calling were performed on a GPU card Nvidia GTX 1070 8 Gb.

2.5 | Pipeline for gDNA Xf detection: detection_script

Reads generated with the two-step debarcoding analysis were analysed using a custom pipeline. In brief, reads were mapped to a database by using Minimap2 software (v. 2.17-r941; Li, 2018) using

--MD and --secondary=no as additional parameters. The mapping was split into two steps: the first step aligned all the reads against a database that includes one representative genome for all sequenced bacterial species (about 11,000 bacterial genomes) present in the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/>), while the second step aligned all reads mapped to Xf in the first step to an Xf-specific database that comprises approximately 60 genomes. For both steps, alignment output files were parsed to retrieve only the best match for reads mapping to multiple genomes. Finally, the number of reads mapping to the same subspecies/strains were combined and summarized in a plot (<https://github.com/lfaingo/xylella>).

2.6 | MLST consensus generation: consensus_script

A second pipeline was written in order to generate MLST consensus after Nanopore sequencing. Briefly, reads from Nanopore sequencing were demultiplexed by using Deepbiner software v. 0.2.0 (Wick et al., 2018). Subsequently, porechop software v. 0.2.4 was used to remove adapters from the kept reads. Two rounds of read-correction by racon software v. 1.3.3 (Vaser et al., 2017) were performed. The corrected reads were passed to jellyfish software v. 2.2.8 (Marçais & Kingsford, 2011) and reads of 100 nucleotides (nt) were generated. These reads were assembled by SPADes software v. 3.12.0 (Bankevich et al., 2012) and the assembled contigs polished by Nanopolish v. 0.11.1 and subsequently by bcftools mpileup software v. 1.7.2 (Danecek & McCarthy, 2017). The reconstructed MLST sequences were compared to all other MLST deposited at <https://pubmlst.org/> (Jolley et al., 2018). The script used for MLST reconstruction can be found at GitHub (<https://github.com/lfaino/xylella>).

3 | RESULTS

3.1 | Detection and identification of Xf in infected plant material by direct Nanopore sequencing

Direct Nanopore sequencing was assessed to detect Xf in naturally infected plants by using the ONT MinION device. The assay was first performed on DNA of 21 olive samples (Set 1) and of seven samples from different plant species (Set 2), making a total of 28 Xf naturally infected samples. All samples were also analysed by real-time PCR, which revealed a variable level of infection among samples. For example, in samples of Set 1, C_t values ranged from 21.28 to 36.17, indicating a difference of about 10^5 times between the most infected (Olive-1) and the least infected (Olive-11) sample (Table 1). The direct Nanopore sequencing was performed in three batches; the first one included samples Olive-1 to Olive-12, the second one samples from Olive-13 to Olive-21 of Set 1 (Table 1), and the third was composed of the seven infected samples from various plant species making up Set 2 (Table 2). All samples were barcoded individually before combining into a single sample for sequencing. The first flowcell generated approximately 3.2 Gb of data for the first batch of 12 samples in a run of 14 hr, the second flowcell produced approximately 2.7 Gb for the remaining nine olive samples in Set 1 in around 30 hr, while the third flowcell generated approximately 5.5 Gb for Set 2 in around 30 hr. All these flowcells were stopped before reaching the 48 hr maximum running time due to pore saturation. Next, the sequencing data batches were subjected to demultiplexing based on the individual barcodes, and we were able to retain approximately 1.3 Gb and 800 Mb of data for the two batches from Set 1 (40% and 30% of the total reads, respectively), and approximately 2 Gb of data for Set 2. The amount of data for the individual samples ranged from approximately 600 Mb in the *Cystus* spp. sample to approximately 10 Mb in Olive-17, illustrating

high variability between samples, even though similar amounts of input gDNA were used (Tables 1 and 2). Each sample was analysed using a custom python script (called detection_script; see Materials and Methods) in order to identify Xf. Twenty-two out of 28 samples showed reads that map uniquely to the genome of *Xylella* spp. (Tables 1 and 2). With the flowcell throughput achieved in our work, all samples with no reads related to the Xf genome had a C_t value >31 in the real-time PCR assays (Tables 1 and 2). To better assess the sensitivity of the direct Nanopore DNA sequencing, we prepared 12 samples of uninfected olive DNA spiked with a known concentration range of DNA of Xfp strain CFBP 8402 close to the detection limit of real-time PCR according Modesti et al. (2017) (Set 3; Table 3). A new Nanopore flowcell was used to sequence the samples of Set 3, generating approximately 667 Mb of data in 22 hr. The amount of data per sample ranged from 45 Mb to 7.9 Mb and Xfp was identified in only four samples using the detection_script (Table 3). These data show that direct DNA sequencing with Nanopore falls short in sensitivity when compared with real-time PCR.

3.2 | Nanopore amplicon sequencing

In order to increase the sensitivity of Nanopore sequencing for the detection of Xf, we pursued PCR-based amplification of Xf-specific targets followed by MinION sequencing of the amplicons. To this end, we used *cysG* and *malF* primer sets as suggested in the PM7/24 (4) (EPPO, 2019b) for the subspecies identification. PCR amplification followed by MinION sequencing was performed on the spiked DNA set (Set 3) to check the assay sensitivity. *cysG* and *malF* amplicons of the spiked samples were tagged with barcodes and sequenced on MinION. The MinION device generated about 290 Mb of data for all 12 samples combined, and after demultiplexing, we retained approximately 155 Mb of data. The number of reads per sample ranged from 14,360 to 54,405 for LOD-10 and LOD-2, respectively (Table 4). The detection_script was used to detect Xf in each sample. The number of reads matching uniquely to a bacterial genome varied from 3.39% in sample LOD-9 to 50.35% in sample LOD-5 (Table 4). As expected, $>95\%$ of those reads mapped to Xf (Table 4, Figure 1). The only exception was sample LOD-9, for which a low number of reads mapped to bacterial genomes, suggesting amplification problems (Table 4, Figure 1). Our findings showed that Nanopore-based detection of Xf based on amplicon sequencing detected the majority of the samples of Set 3, including samples (from LOD-7 to LOD-12) that were not correctly detected or were not reproducible by real-time PCR (Table 4).

We reasoned that by correcting Nanopore sequencing errors, because the same region is sequenced multiple times, we can produce a more accurate sequence that allows us to identify Xf at the subspecies level. To validate this, we used the detection_script in combination with a specific Xf database formed by every Xf genome available in the NCBI database (c.60 genomes). For all 11 positive samples of Set 3, $>95\%$ of the reads correctly mapped to a genome of Xfp (Figure 2). To further validate this approach, the same

Sample	Mb	No. of reads	No. of reads mapping to bacteria (%) ^a	No. of reads mapping to <i>X. fastidiosa</i> (%) ^b
LOD-1	1.95	42,384	16,543 (39.0)	16,367 (98.9)
LOD-2	2.50	54,405	26,119 (48.0)	25,870 (99.0)
LOD-3	1.00	21,805	7,765 (35.6)	7,689 (99.0)
LOD-4	1.37	29,806	12,039 (40.4)	11,866 (98.6)
LOD-5	1.72	37,384	18,827 (50.4)	18,683 (99.2)
LOD-6	1.89	40,984	7,347 (17.9)	7,163 (97.5)
LOD-7	1.04	22,675	3,147 (13.9)	2,794 (88.8)
LOD-8	1.02	22,087	5,403 (24.5)	4,669 (86.4)
LOD-9	1.20	24,336	832 (3.4)	2 (0.2)
LOD-10	0.66	14,360	4,687 (32.6)	4,394 (93.7)
LOD-11	0.68	14,710	4,867 (33.1)	4,659 (95.7)
LOD-12	1.21	26,334	12,336 (46.8)	12,124 (98.3)
Control ^c	NA	4,987	—	0 (0)

^aPercentage of reads mapped to bacterial species over the number of total reads sequenced.

^bPercentage of reads mapped to *X. fastidiosa* over the number of reads mapped to bacterial species.

^cNoninfected *Olea europaea*.

TABLE 4 Nanopore amplicon sequencing of *cysG* and *malF* amplified from DNA of healthy olive samples spiked with known amounts of *Xylella fastidiosa* subsp. *pauca* strain CFBP 8402 DNA (data Set 3)

FIGURE 1 Stacked bar charts showing the relative abundance of bacterial species compared with *Xylella fastidiosa* using the detection_script on Nanopore amplicon sequencing of olive DNA spiked with known concentrations of *X. fastidiosa* subsp. *pauca* DNA (Set 3)

procedure (amplification of *cysG* and *malF*, Nanopore sequencing, querying the Xf-customized database) was used on samples of Sets 2 and 4, the gDNA from different naturally infected plant species and uninfected samples, respectively (Table 5, Figure 3). For all samples in Set 2, except for *R. alaternus*, more than 35% of the amplicon-derived reads mapped to Xf (Table 5). Additionally, the analysis using the Xf-specific database showed that >90% of the reads mapped to the Xfm genome, including *R. alaternus* (Figure 3). No Xf reads were identified by sequencing the amplicons of the

healthy samples (Set 4; Table 5). To further support the subspecies identification results, we generated a consensus for the *cysG* and *malF* sequences using the consensus_script. These consensus sequences were aligned to the Xf-specific database, matching 100% to the *multiplex* subspecies (Figure 3). This identification corresponds to the recent study of Marchi et al. (2018) on the occurrence of Xfm in Tuscany. These results highlight that Nanopore amplicon sequencing can detect Xf in naturally infected samples at subspecies level.

FIGURE 2 Stacked bar charts showing the relative abundance of *Xylella fastidiosa* subsp. *pauca* compared to other *X. fastidiosa* subspecies using the detection_script on Nanopore amplicon sequencing of olive DNA spiked with known concentrations of *X. fastidiosa* subsp. *pauca* DNA (Set 3). The most abundant subspecies is *X. fastidiosa* subsp. *pauca*

TABLE 5 Nanopore amplicon sequencing of *cysG* and *malF* amplified from DNA of naturally infected plants samples collected in Tuscany region, Italy (data Set 2) and from healthy samples of different plant species collected in Latium region (data Set 4)

Sample	H/I ^a	C _t ^b	Mb	No. of reads	No. of reads mapping to <i>Xylella fastidiosa</i> (%) ^c
<i>Prunus amygdalus</i>	I	27.4	0.28	6,108	3,396 (55.6)
<i>Rhamnus alaternus</i>	I	NA	0.44	9,462	28 (0.3)
<i>Cystus</i> sp.	I	25.8	0.44	9,600	5,656 (58.9)
<i>Rosmarinus officinalis</i>	I	35.1	0.29	6,228	2,178 (35.0)
<i>Lavandula</i> sp.	I	28.8	1.19	25,798	10,391 (40.3)
<i>Polygala myrtifolia</i>	I	25.1	0.51	11,085	7,065 (63.7)
<i>Spartium junceum</i>	I	18.6	0.50	10,959	5,633 (51.4)
<i>Prunus amygdalus</i>	H	NA	NA	5,620	0 (0)
<i>Rhamnus alaternus</i>	H	NA	NA	3,718	0 (0)
<i>Cystus</i> sp.	H	NA	NA	3,446	0 (0)
<i>Rosmarinus officinalis</i>	H	NA	NA	5,857	0 (0)
<i>Lavandula</i> sp.	H	NA	NA	3,895	0 (0)
<i>Polygala myrtifolia</i>	H	NA	NA	3,487	0 (0)

^aI, infected; H, healthy.

^bCycle threshold obtained by Francis et al. (2006), TaqMan version.

^cPercentage of reads mapped to *X. fastidiosa* over the number of total reads sequenced.

3.3 | Identification of Xf ST using Nanopore amplicon sequencing

It is generally accepted that the ST of Xf can be determined by assessing seven housekeeping genes (*leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, *gltT*) in an MLST analysis (PM 7/24 (4)) (EPPO, 2019b). We therefore pursued typing the Xf infections based on these seven genes upon sequencing of PCR-generated amplicons. To this end, we first amplified DNA fragments using primer pairs for all housekeeping genes

using, as template, DNA from *Cystus* sp., *R. officinalis*, and *Lavandula* sp. of Set 2 and subsequently sequenced them using Nanopore sequencing. The generated sequences were used to make consensus for all seven fragments for these plant species. Each fragment for all seven housekeeping genes for the same three plant samples was also Sanger sequenced. These plant species were selected because the ST of Xf had not previously been determined (Saponari et al., 2019). The consensus_script was used to generate a consensus sequence using only Nanopore reads. The script generated seven

FIGURE 3 Stacked bar charts showing the relative abundance of different *Xylella fastidiosa* subspecies using the detection_script on Nanopore amplicon sequencing of DNA from different plant species (Set 2). The most abundant subspecies is *X. fastidiosa* subsp. *multiplex*

FIGURE 4 Heatmap representing sequence alignments of seven concatenated multilocus sequence typing (MLST) for 87 sequence types (STs) deposited at the MLST database (<https://pubmlst.org>), three sequences derived from Sanger sequencing of *Rosmarinus officinalis*, *Cystus* sp., and *Lavandula* sp., and three sequences derived from consensus of the Nanopore sequencing for *R. officinalis*, *Cystus* sp., and *Lavandula* sp. plant samples

sequences for each sample, one for each of the housekeeping genes, that were subsequently compared with the same sequences generated by Sanger technology (Yuan et al., 2010), followed by querying the database at <https://pubmlst.org/organisms/xylella-fastidiosa>

(Jolley et al., 2018). Pairwise alignment of concatenated sequences of all seven MLST derived from Sanger and Nanopore consensus revealed 100% identity (Figure 4), demonstrating the robustness of the Nanopore sequencing-based approach. Additionally, when the

concatenated sequences derived from both Sanger and Nanopore sequencing were compared to the Xf MLST database, we found that samples of *Cystus* sp., *R. officinalis*, and *Lavandula* sp. showed the following MLST profile: *leuA*_5, *petC*_3, *malF*_5, *cysG*_3, *holC*_3, *nuoL*_21, *gltT*_3. The combination of the seven alleles corresponded to ST 87 (Figure 4), which is in agreement with recent findings of Xf ST 87 in *P. amygdalus*, *P. myrtifolia*, *S. junceum*, and *R. alaternus* (Saponari et al., 2019). Altogether, our data show that Nanopore amplicon sequencing can be used in infected plants to detect Xf, and at the same time determine subspecies and ST.

4 | DISCUSSION

Over the last 15 years, NGS has transformed genomic research, being applied to genomics, transcriptomics, metagenomics, and diagnostics (Charalampous et al., 2019; Cook et al., 2019; Jain et al., 2018; Leggett et al., 2019). These technologies have recently been applied to the diagnosis of plant pathogens (Badial et al., 2018; Chalupowicz et al., 2019), including the detection and identification of Xf (Bonants et al., 2019). Although several diagnostic methods are available for the detection of Xf (EPPO, 2019b), the identification of subspecies and ST are currently laborious and time-consuming. Recently, a tetraplex real-time PCR assay was developed for simultaneous detection and identification of Xf subspecies in plant tissues (Dupas et al., 2019). The detection of Xf at subspecies level is fundamental, and in the case of new outbreaks or new plant hosts, the identification of the ST is strongly recommended (EPPO, 2019b).

To circumvent the complexity of the detection and identification of Xf subspecies and ST, we investigated the use of the ONT MinION device. One of the advantages of the MinION device compared to other NGS platforms is its portability. In this study, direct Nanopore DNA sequencing was assessed for the first time by using naturally infected plant samples (Sets 1 and 2). However, our results showed that the bacterium can only be reliably detected, and its subspecies identified, in samples with high levels of Xf. Every sample lacking reads mapping to the Xf genomes showed a high C_t value in the real-time PCR that, associated with low sequencing throughput, made Xf undetectable. The lack of detection in samples with low DNA bacterial concentrations was confirmed by using artificially spiked olive samples with Xf DNA concentrations close to the limit of detection with real-time PCR (Set 3), which is currently considered the most sensitive assay for Xf detection (EPPO, 2019b; Modesti et al., 2017). Nanopore direct DNA sequencing of these samples provided only a low number of reads, resulting in an even lower number of reads mapping to Xf genomes, making the analysis questionable. A similar result was obtained by Bonants et al. (2019) using Illumina sequencing, as they reported the ability of NGS to determine the ST of Xf only in samples with high bacterial concentration. Moreover, Chalupowicz et al. (2019) discussed the applicability of Nanopore sequencing on artificially infected plant material with symptoms. They reasoned that Nanopore sequencing is applicable on heavily infected plant samples, where the pathogen:plant DNA ratio is relatively high,

allowing the sequencing of pathogen DNA even with low throughput, suggesting a further investigation on symptomless plants. The low sensitivity of the direct Nanopore sequencing may be due to the low throughput generated for each sample, which can imply a low quality and quantity of the DNA in the tested samples. It is worth mentioning that Nanopore sequencing suffers from low throughput when the DNA used for the sequencing has contaminants and/or when the input DNA is made of short fragments (Chalupowicz et al., 2019). Further optimization of the DNA purification and addition of a size selection step may be necessary to maximize the performance of the Nanopore system. This aspect is of importance because Xf has a very broad host range and the DNA quality can be compromised by contaminants of the host matrix. Charalampous et al. (2019) developed a DNA extraction in order to deplete host DNA from the samples and thus enrich it for pathogen DNA. Using this approach, they were able to identify respiratory pathogens in human samples. However, carrying out massive analyses in practice is not compatible with time-consuming or expensive DNA purifications, as generally neither the DNA concentration, nor the DNA quality, are routinely determined in official analysis.

An alternative to employing sophisticated DNA extraction methodology is amplicon sequencing (Kilianski et al., 2015; Radhakrishnan et al., 2019). In our study this approach was assessed in order to overcome the low throughput of the flowcells and to set up a more reliable and sensitive detection method, based on the amplification of two housekeeping genes followed by Nanopore sequencing. Sequencing of *cysG* and *malF* for subspecies discrimination is suggested (Dupas et al., 2019; EPPO, 2019b). Nanopore amplicon sequencing was efficacious for Xf detection of the spiked samples with low concentrations (<10 fg/PCR) of Xf target DNA (Set 3). The addition of the amplification step, even though it makes the procedure longer, has several advantages: (a) reduction of the influence of poor DNA quality/quantity; (b) mitigation of sequencing errors by increased sequence depth; and (c) production of useful data faster than genomic DNA sequencing. The amplification step allows the detection of the pathogen as well as the identification of its subspecies and ST in a single sequencing step. The results of amplicon sequencing of Set 2 on *S. junceum*, *P. myrtifolia*, *R. officinalis*, *Lavandula* sp., *P. amygdalus*, and *Cystus* sp. confirmed previous findings that identified the subspecies *multiplex* in the plant infections (Marchi et al., 2018; Saponari et al., 2019). Moreover, amplicon sequencing performed on the seven MLST fragments in samples of *R. officinalis*, *Cystus* sp., and *Lavandula* sp. confirmed the recent findings that assigned the ST87 to the subsp. *multiplex* recovered in Tuscany (Saponari et al., 2019). The Nanopore amplicon sequencing workflow allows the detection of Xf and the identification of its subspecies and ST, producing the data output in a few hours and avoiding the time-consuming procedure required for Sanger sequencing of the two/seven housekeeping gene fragments (e.g., amplicon purification from agarose gel, Sanger sequencing, sequence purification, and comparison in data bank, analyses in the *Xylella* data bank for ST assignment). ST characterization is essential when an infection in a pest-free area or in a new plant species is detected. The ST determination can highlight

possible recombination between strains that affect the evolution and adaptation to new plant hosts and suggest information on the origin of the strains (Coletta-Filho et al., 2017; Denancé et al., 2017; Scally et al., 2005).

The evidence obtained in this study shows that the sequencing of two or seven housekeeping genes by MinION is a reliable and robust method to detect and identify Xf from infected plant material, even with low bacterial concentrations, representing a powerful tool for a rapid Xf diagnosis. This approach is of interest for Xf detection in trade plants, and for latently infected material that represents one of the most serious threats for Xf dissemination. For an "in field" application, further studies are required to make direct Nanopore sequencing on genomic DNA reliable for detecting low DNA bacterial concentrations, for instance by using single flow-cells for the processing of individual samples. This allows a greater depth of sequencing, and consequently a higher possibility of detection and identification of Xf/subspecies/ST. In conclusion, the results obtained in this study pave the way for novel opportunities for Nanopore sequencing as an effective surveillance tool for early, sensitive, reliable, and robust detection of Xf and other fastidious pathogens.

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DATA AVAILABILITY STATEMENT

All data were deposited at the NCBI (<https://www.ncbi.nlm.nih.gov/>) under the accession number PRJNA575828 while all scripts used in the paper are deposited at <https://github.com/lfaino/xylella>.

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