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Complete genome assemblies of several *Xylella fastidiosa* subspecies *multiplex* strains reveals high phage content and novel plasmids

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Summary. The Gram-negative bacterium *Xylella fastidiosa* (*Xf*) was originally found in the Americas, but has now been identified in more than 20 countries across America, Asia, and Europe. This plant pathogen is currently listed as a priority pest in Europe due to its socio-economic and ecological impacts. Within the three *Xf* subspecies *fastidiosa*, *multiplex* and *pauca*, subsp. *multiplex* displays a notably wider range of host plants than the other two subspecies. Comparative genomics may allow determination of how *Xf* subsp. *multiplex* adapts to new and diverse hosts and environments, so it is important that more genomes of this subspecies are defined. Twelve complete closed genomes sequences of *Xf* subsp. *multiplex* were obtained using a hybrid assembly approach combining Illumina and Oxford Nanopore technologies. The combined use of Canu and Unicycler assemblers enabled identification and closure of several plasmid sequences with high similarity to other plasmids described in strains of *Xf* subsp. *fastidiosa* and subsp. *pauca*. The analysis also revealed prophage sequences and contigs outside the chromosomes, annotated as phages. These new genomes, in conjunction with those existing in GenBank, will facilitate exploration of the evolutionary dynamics of *Xf* subsp. *multiplex,* its host adaptation mechanisms, and the potential emergence of novel strains of this important plant pathogen.

Keywords. Hybrid assembly, prophages, quarantine phytopathogen.

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INTRODUCTION

Xylella fastidiosa is a fastidious, Gram-negative, xylem-limited bacterium in the *Xanthomonadaceae*, which is a major transcontinental plant health threat, with serious socioeconomic impacts. The bacterium causes diseases on a wide range of agricultural crops, ornamental and landscape plants, and plants with cultural and heritage values (EFSA *et al.*, 2023). This Gram-negative bacterium affects many plant species, leading to symptoms such as leaf scorching, wilting, decline, and complete canopy death. Some important

diseases caused by *X. fastidiosa* include Pierce's Disease (PD) of grapevine, Citrus Variegated Chlorosis (CVC), Almond Leaf Scorch (ALS), and Olive Quick Decline Syndrome (OQDS) (EFSA *et al.*, 2023). This bacterium can also severely impact urban trees (Sherald *et al.*, 1987; Harris *et al.*, 2014; Desprez-Loustau *et al.*, 2021), and plants within natural environments (Denancé *et al.*, 2017). The range of hosts susceptible to *X. fastidiosa* continues to expand, with over 690 plant species across 306 genera and 88 families as hosts (EFSA *et al.*, 2023).

Although *X. fastidiosa* has allopatric origins in the Americas (Almeida and Nunney, 2015; Vanhove *et al.*, 2019), its current distribution is now in more than 20 countries in the Americas, Asia, and Europe (EFSA *et al.*, 2023; EPPO, 2023). In Europe, *X. fastidiosa* emerged as an important pathogen in 2013, associated with a severe epidemic in olive trees in Italy. This epidemic, associated to OQDS, is ongoing, causing the loss of hundreds of olive trees annually. European territories have witnessed other *X. fastidiosa* outbreaks impacting mainly native plant species in natural environments and ornamentals in France and Portugal, while Spain has faced epidemics mainly affecting almonds and grapes (Velasco-Amo *et al.*, 2022; EFSA *et al.*, 2023). Sánchez *et al.* (2019) ranked *X. fastidiosa* amongst first priority pests for the European Union (EU) when considering economic, social, and environmental domains. These authors estimated, in a scenario where *X. fastidiosa* would spread extensively across Europe, that potential annual costs would exceed €5.5 billion. This accounted for potential losses in the olive, almond, citrus, and grape sectors, reflecting the substantial economic impacts that this pathogen could cause (Sanchez *et al.*, 2019).

Xylella fastidiosa is a genetically diverse bacterium that includes three main subspecies: subspp. *fastidiosa, multiplex* and *pauca;* although other subspecies have been proposed (Schaad *et al.*, 2004; Schuenzel *et al.*, 2005; Denancé *et al.*, 2019). These subspecies can be further grouped below subspecies level into Sequence Types (ST), based on Multilocus Sequence Typing (MLST) analyses (Yuan *et al.*, 2010). The subspecies *multiplex* has gained particular attention due to its ability to infect a diverse range of hosts, as a damaging and adaptable pathogen. Considering the EFSA list of confirmed *X. fastidiosa* hosts for which molecular-characterization typing approaches have been carried out to characterize subspecies, approx. 62% of the records were for plants infected by subsp. *multiplex*, while 17.1% subsp. *fastidiosa* and 16.6% by subsp. *pauca* (EFSA *et al.*, 2023).

Complete genome sequences are important for describing the biology of plant pathogens, identifying virulence factors, and understanding genetic diversity, potential origins, and introductory pathways (Landa *et al.*, 2019). Comparative genomics, facilitated by multiple genomes of different strains and subspecies, offers a powerful means to explore the evolutionary dynamics of *X. fastidiosa*, its adaptation to new hosts, and the emergence of novel strains (Potnis *et al.*, 2019; Vanhove *et al.*, 2019; Castillo and Almeida, 2023).

Despite the advances made in *X. fastidiosa* genomics, understanding of *X. fastidiosa* subsp. *multiplex* at the genomic level remains limited. Several genomes from other *X. fastidiosa* subspecies are available, but scarcity of complete genomes from *multiplex* restricts ability to comprehensively study the genetics, biology, and evolution of this pathogen. This underscores the urgent need for an expanded dataset of *X. fastidiosa* subsp. *multiplex* genomes.

The present study has provided the genome sequences of 12 strains of *X. fastidiosa* subsp. *multiplex*, from different host plants in different countries, and shows the significance of acquiring additional genomes of this subspecies as a critical step in advancing understanding of this versatile and destructive plant pathogen.

MATERIALS AND METHODS

Table 1 shows the 12 strains of *X. fastidiosa* subsp. *multiplex* sequenced in this study. These strains are deposited at the *X. fastidiosa* collection of the Institute for Sustainable Agriculture (IAS-CSIC), Córdoba, Spain. Strains XYL466/19, XYL468 and Santa29b belonging to the ST81 were isolated from leaf petioles of *Olea europaea* var. *sylvestris* and *Santolina chamaecyparissus* on periwinkle wilt-modified (PW) (for XYL466/19 and XYL468) and PD2 (for Santa29b) solid media, following the EPPO isolation procedures (EPPO, 2023). The remaining strains were provided by researchers from different laboratories, or were acquired at the CIRM-CFBP collection of plant-pathogenic bacteria, INRAE, France (Table 1).

The strains were grown in PD2 solid medium at 28˚C in the dark during 7 to 12 days (depending on the strain). Genomic DNA was extracted using the Quick DNA Fungal/Bacteria Miniprep kit (Zymo Research Group). The integrity of DNA was measured by gel electrophoresis and concentrations were estimated using a spectrofluorometer (Qubit; Thermo Fisher Scientific).

Illumina sequencing libraries were prepared following manufacturer recommendations, and were sequenced using the platforms Hiseq 4000 at the StabVida sequencing facility, Caparica, Portugal (for strains XYL466/18 and XYL468), or iSeq 100 at the

Table 1. Information related to the strains belonging to *Xylella fastidiosa* subspecies *multiplex* used in this study.

Strain	Other names	ST[a]	Host	Geographical origin	Isolation Year
CFBP8417	LSV 46.78	6	*Spartium junceum*	France: Alata, Corsica	2015
CFBP8418	LSV 46.79	6	*Spartium junceum*	France: Alata, Corsica	2015
CFBP8070	GA Plum LSV 40.38	10	*Prunus* sp.	USA: Georgia	2004
CFBP8075	LSV 42.30	27	*Prunus* sp.	USA: California	unknown
CFBP8068	ATCC35873, 2687 ELM-1, LSV 00.54	41	*Ulmus americana*	USA: Washington	unknown
CFBP8173	LSV40.39, ICPB50039, Hopkins PL788, Wells2679, ATCC228771, ATCC35871, ICMP15199, ICMP8735, ICMP8746, LMG9063, Labo13350, Labo13352, Labo13355, NCPPB4431	41	*Prunus salicina*	USA: Georgia	unknown
Santa29b		81	*Santolina chamaecyparissus*	Spain: Alcafar, Menorca	2022
XF3348		81	*Prunus dulcis*	Spain: Binissalem, Mallorca	2018
XYL1752		81	*Prunus dulcis*	Spain: Ciudatella, Menorca	2017
XYL1966/18		81	*Olea europaea*	Spain: Ciudatella, Menorca	2018
XYL466/19		81	*Olea europaea* var. *sylvestris*	Spain: Sant Llorenç, Mallorca	2019
XYL468		81	*Olea europaea* var. *sylvestris*	Spain: Manacor, Mallorca	2019

[a] Sequence Type (ST) was determined by MLST analysis or by BLAST search of whole genome against the *Xylella fastidiosa* MLST database (https://pubmlst.org/xfastidiosa/; accessed on 02 November 2022).

IAS-CSIC facility (for strains CFBP8068, CFBP8070, CFBP8075, CFBP8173, and Santa29b). For the remaining strains, Illumina data were retrieved from the Sequence Read Archive (SRA) database: Strains CFBP8417 (SRR8454254), CFBP8418 (SRR8454358), and XYL1966/18 (SRR11931336). Illumina reads were trimmed and filtered with the fastp tool v0.23.2 (Chen *et al.*, 2018). Before the assembly process, resulting fastq files were analyzed with Krakren2 v2.1.2 (Wood *et al.*, 2019) using the PlusPFP v.6-5-2023 database (https://benlangmead.github.io/aws-indexes/k2) to identify and remove any contamination from the reads that may have been introduced during the library preparation, and to only retain reads assigned to the *X. fastidiosa* taxon.

Oxford Nanopore Technologies (ONT) sequencing libraries were prepared by multiplexing, using the ligation sequencing gDNA and Native barcoding kit SQK-NBD114.24 or the VolTRAX Multiplex Kit VMK004 in the VolTRAX v0.21.0 system. These libraries were loaded in, respectively, R9.4.1 or R10.4.1 flow cells, in a MK1C v6.0.7 sequencing device. ONT sequencing reads were basecalled with Guppy v6.4.2, and were trimmed with Porechop v0.2.4 (Wick *et al.*, 2017).

Long-read *de novo* genome assemblies were carried out using Canu v2.2. The draft genomes obtained were subsequently polished using the Illumina high-quality short-reads (Q > 25), first using Polypolish v0.5.0 (Wick and Holt, 2022), and then two rounds with POLCA v4.0.9 (Zimin and Salzberg, 2020). Two of the *X. fastidiosa* genomes required an additional step, which involved scaffolding and direction of contigs with a reference genome (strain IVIA5901 from *X. fastidiosa* subsp. *multiplex*). This was carried out using RagTag v2.1.0 (Alonge *et al.*, 2022), and gap filling was then carried out using TGS-GapCloser 1.0.3 (Xu *et al.*, 2020) with Racon v1.4.20. Final assemblies were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova *et al.*, 2016) before submission to GenBank.

A phylogenetic analysis using a total of 111 *X. fastidiosa* whole genomes belonging to subsp. *multiplex* was carried out including all the genomes available at the GenBank database (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/173/), in combination with the 12 complete genomes obtained in this study. The genomes were annotated with prokka v1.14.6 (Seemann, 2014) with default parameters, and coding sequences (CDSs) were used to estimate the core genome with CoreCruncher using the MAFFT (Katoh and Standley, 2013) algorithm to build the core genome alignment. Following this, ambiguous sequences or poorly aligned regions were eliminated from the multiple sequence alignment using ClipKIT v 1.3. A maximum likelihood (ML) tree was then constructed with IQtree v2.2.0 using the GTR+I+G4 substitution model determined by ModelTest-NG c0.1.6 (Nguyen *et al.*, 2015; Darriba *et al.*, 2020), and was plotted with ggtree v3.6.2 (Yu *et al.*, 2017). Strain IVIA5235 of subsp. *fastidiosa* was used as an outgroup. Tree topology was midpoint rooted, and branch support was assessed using 1,000 bootstrap replicates.

RESULTS AND DISCUSSION

The hybrid sequencing and assembly approach allowed reconstruction and circularization of the complete genomes of ten of the strains (Table 2). The genome of strains XYL466/18 and XYL468 resulted in scaffolds due to conflicting regions.

Three complete plasmids were identified and assembled in strain CFBP8070, designated as pXF-P1. CFBP8070 (43,491 bp), pXF-P2.CFBP8070 (26,328 bp), and pXF-P3.CFBP8070 (1,286 bp) (Table 2). Johnson *et al.* (2023) highlighted the challenges that long-read assemblers like Canu faced for detecting bacterial plasmids. To reconstruct and close the plasmids in the CFBP8070 strain, a combination of Canu and Unicycler assemblers was necessary. Results of BLASTN revealed similarities with previously-described plasmids. Plasmid pXF-P1.CFBP8070 exhibited a 96% similarity, covering less than 80% of the query, with plasmid pXF51ud from strain U24D, a member of subsp. *pauca* isolated from a citrus plant in Brazil (Pierry *et al*., 2020). Plasmid pXF-P2.CFBP8070 displayed 99% similarity, covering the entire query, with plasmid pXF26-Oak35874 from strain Oak35874, belonging to subsp. *multiplex* and isolated from *Quercus* in Washington DC, United States of America (USA: O'Leary and Burbank, 2023). Plasmid pXF-P3.CFBP8070 showed 90% similarity with 99% coverage to plasmid pUCLAb from strain UCLA, of subsp. *fastidiosa* isolated from grapevine in California, USA (Guilhabert *et al*., 2006).

The discovery of identical plasmids in distinct *X. fastidiosa* subspecies further supports the possibility of horizontal gene transfer (HGT) among bacterial strains (Rogers and Stenger, 2012). HGT and recombination are key factors in the emergence of new strains capable of colonizing new hosts (Burbank and Van Horn, 2017). This phenomenon could elucidate the high ability of subsp. *multiplex* to infect a wide range of host plants.

A Maximum-likelihood phylogenetic tree was constructed showing the different STs, hosts, and geographical origins of the strains included, which agreed with those of described in previous studies (Denancé *et al*., 2017; Landa *et al*., 2019; Dupas *et al*., 2023) (Figure 1). As previously reported, strains identified as belonging to ST6 from France and Spain were polyphyletic (Landa *et al*., 2019; Dupas *et al*., 2023), with strains belonging to ST7 from France clustering together with those from the USA from the same ST, and closer to Spanish ST6 strains. In contrast, French ST6 strains clustered with Dixon strain from ST6, isolated from almonds in California, and closer to a subgroup formed by ST81 strains from Spain. These ST81 strains clustered with Fillmore and Riv5 strains from California, USA, from the same ST, which suggests a potential introduction of ST81 strains from the USA into the Balearic Islands (Moralejo *et al*., 2020).

The remaining *X. fastidiosa* strains, including strains from Italy belonging to ST87 and four of the strains sequenced in this study, were grouped according to their ST in more ancestral clades. These clades include ten strains isolated in Italy from the host plants *Polygala myrtifolia, Prunus dulcis, Rhamnus alaternus* and *Spartium junceum*, one strain isolated in Brazil from *Prunus domestica,* and 20 strains isolated in the USA from *Lupinus, Platanus*, *Prunus*, *Quercus*, *Ulmus*, *Vaccinium* and *Vinca* sp. All the strains within these ancestral clades were primarily isolated from the southeastern USA, with two exceptions: two strains isolated from *Prunus* in California, CFBP8075 assigned to ST27 and ICMP8739 assigned to ST41 (Kant *et al*., 2023), and the strain RAAR14 plum327 from Brazil from ST26. This further supports the hypothesis that subsp. *multiplex* likely originated in the southeastern USA (Landa *et al*., 2019).

The presence of *X. fastidiosa* strains in California, deviating from typical geographical distribution, prompts consideration. Those strains were sourced from different bacterial collections and could signify introductions from the southeastern USA. However, data inaccuracies could have occurred during strain documentation in the collection database, or errors may have occurred during handling, as these have occurred in the past (Nunney *et al*., 2012), and at least one of the strains was isolated in 1985. This underscores the necessity of providing precise metadata when depositing microorganisms into culture collections. It also emphasizes the significance of open sharing and preserving genome data associated with respective correct metadata to ensure accuracy and reliability (Nunney *et al*., 2012; Sabot, 2022).

Annotation of *X. fastidiosa* subsp. *multiplex* genomes with the RAST server (Aziz *et al*., 2008) revealed the presence of prophage sequences and phage contigs within most of the bacterial chromosomes assessed in the present study (Table 2). Strains isolated from Menorca Island exhibited three phage sequences, in contrast to the strains isolated from Mallorca Island. Both of these islands are in the Balearic Archipelago in Spain, where no phage sequences were annotated. Despite belonging to the same subspecies and ST81, strains from Menorca Island were proposed to have been introduced from Mallorca Island (Moralejo *et al*., 2020). Among the strains analyzed, strain CFBP8418 displayed two contigs annotated as phages, while strains CFBP8070 and CFBP8417 each presented one contig annotated as phages.

Table 2. General genome features and assembly statistics for strains of *Xylella fastidiosa* subspecies *multiplex* used in the study.

Strain	Number of contigs	Contigs type	Size (bp)	Assembly	Total Cove-rage (X)	Contami-nation (%) [a]	Complete-ness (%) [a]	GC content (%)	Number of CDS [b]	Number of tRNA	Number of rRNA	Number of ncRNA	Accession number	Additional contigs [c]	Size of additional contigs (bp) [c]
CFBP8417	1	Chr	2,672,285	Canu	180	0.19	99.08	51.9	2,423	49	6	4	CP136980	1	44,569
CFBP8418	1	Chr	2,692,457	Canu	128	0.19	99.08	51.9	2,460	49	6	4	CP136979	2	22,299 32,325
CFBP8070	4	Chr pXF-P1.CFBP8070 pXF-P2.CFBP8070 pXF-P3.CFBP8070	2,796,854 43,491 26,328 1,286	Canu Unicycler Unicycler Unicycler	360	0.31	98.98	52.1	2,686	49	6	4	CP136975 CP136976 CP136977 CP136978	1	42,466
CFBP8075	1	Chr	2,439,524	Canu	115	0.19	99.03	51.9	2,085	49	6	4	CP136974	-	-
CFBP8068	1	Chr	2,548,602	Canu	904	0.19	99.06	51.9	2,233	49	6	4	CP136973	-	-
CFBP8173	1	Chr	2,548,622	Canu	105	0.19	99.06	51.9	2,243	49	6	4	CP136972	-	-
Santa29b	1	Chr	2,593,199	Canu	213	0.19	99.08	52.7	2,313	49	6	4	CP136971	3	35,805 35,815 35,805
XF3348	1	Chr	2,682,254	Canu	1,110	0.19	99.08	52.1	2,426	49	6	4	CP136970	-	-
XYL1752	1	Chr	2,769,601	Canu	847	1.88	99.08	52.3	2,541	50	6	4	CP136969	3	40,489 42,412 42,428
XYL1966/18	1	Chr	2,708,059	Canu	511	0.19	99.08	52.2	2,453	49	6	4	CP136968	-	-
XYL466/19	2	Chr-Scaffold	2,790,648	Canu	1,026	0.19	99.09	52.3	2,552	49	6	4	CP136967	-	-
XYL468	2	Chr-Scaffold	2,809,670	Canu	787	0.19	99.08	52.2	2,610	49	6	4	CP136966	-	-

[a] Genome Contamination (Redundancy) and Completeness were checked with checkM (Parks *et al.*, 2014).
[b] Coding sequence (CDS) were annotated with NCBI Prokaryotic Genome Annotation Pipeline.
[c] Contigs annotated as candidate phage with RAST server (Aziz *et al.*, 2008).

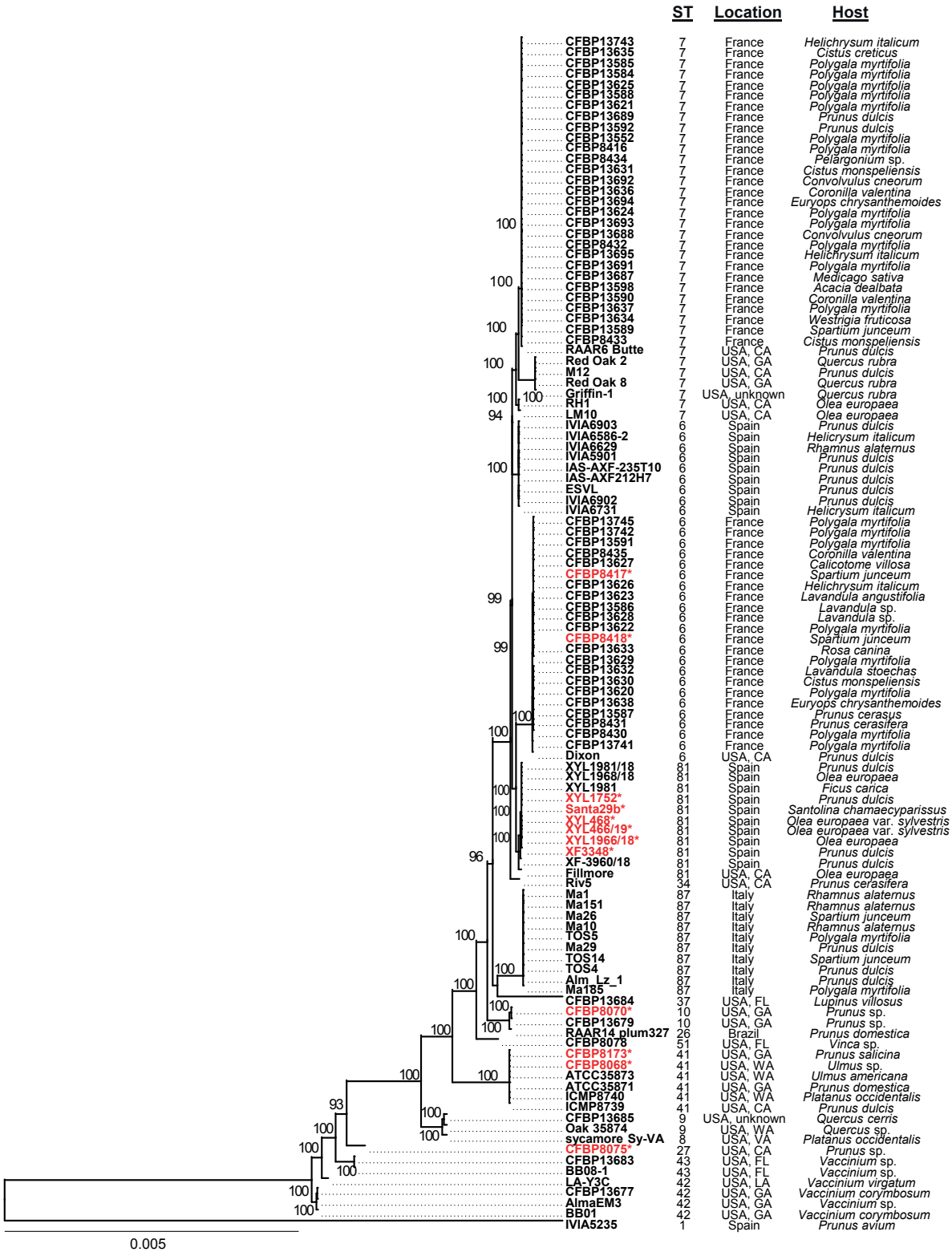

Figure 1. Maximum-likelihood phylogenetic reconstruction of the core genome for 111 *Xylella fastidiosa* subsp. *multiplex* strains. The strain IVIA5235 belonging to *X. fastidiosa* subsp. *fastidiosa* ST1 was used as the outgroup. Sequence type was known and/or confirmed by genome query at the *Xylella fastidiosa* pubMLST database (Jolley *et al.*, 2018), and location and host of isolation are provided. Strains sequenced in this study are shown in red accompanied with an asterisk. Numbers indicate bootstrap values.

Previous studies have documented the existence of prophages and phages in strains of *X. fastidiosa* across different subspecies, associating the presence of prophages with genomic rearrangements and strain divergence (Varani *et al*., 2008, 2013; O'Leary *et al*., 2022). Absence of observable plaques (calvus) on the culture media where these strains grow suggests that the assembled contigs may correspond to phages in lysogenic states (Chen and Civerolo, 2008). The high prevalence of these sequences in strains from subsp. *multiplex* is particularly notable. This prompts the need for further investigation to comprehensively elucidate their significance, the underlying reasons for their abundance, and to provide insights into the mechanisms and potential implications of these sequences for *X. fastidiosa* biology and evolution.

DATA AVAILABILITY STATEMENT

The complete genome sequences of *X. fastidiosa* subsp. *pauca* were deposited at NCBI under BioSample accession numbers: SAMN37751223 (CFBP8417), SAMN37751224 (CFBP8418), SAMN37751225 (CFBP8070), SAMN37751226 (CFBP8075), SAMN37751227 (CFBP8068), SAMN37751228 (CFBP8173), SAMN37751229 (Santa29b), SAMN37751230 (XF3348), SAMN3775131 (XYL1751/17), SAMN37751232 (XYL1966/18), SAMN37751233 (XYL466/19), and SAMN37751234 (XYL468). All these accession numbers are associated with BioProject PRJNA 1026562.

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