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Ecology and Epidemiology

Isolation, Phylogenetic Inferences, and Early Diversification of *Xylella fastidiosa* subsp. *fastidiosa* in Cova da Beira Region, Portugal

Eva Garcia,[1,2] Alexandra K. Kahn,[3] Cristina Rodrigues,[2] Alexandra Camelo,[1,4] Christophe Espírito-Santo,[1,4] Helvecio D. Coletta-Filho,[5] Rodrigo P. P. Almeida,[3] and Joana Costa[1,2,†]

[1] Centre for Functional Ecology, Associate Laboratory TERRA, Department of Life Sciences, University of Coimbra, Coimbra, Portugal
[2] Laboratory for Phytopathology, Instituto Pedro Nunes, Coimbra, Portugal
[3] Department of Environmental Science, Policy, and Management, University of California-Berkeley, Berkeley, CA 94720, U.S.A.
[4] CATAA - Centro de Apoio Tecnológico Agro-Alimentar, Castelo Branco, Portugal
[5] Centro APTA Citros Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil
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Abstract

The introduction of *Xylella fastidiosa*, a plant pathogen with a broad host range, poses a significant threat to agriculture and natural ecosystems. We analyzed six *X. fastidiosa* subsp. *fastidiosa* strains obtained from the Beira Interior region of Portugal, an area known for fruit production. Whole-genome sequencing and phylogenetic analyses were performed to genetically characterize these strains and determine their origin. The results suggest that these outbreak-demarcated areas originated from a single introduction event traced back to California, United States. All six strains belong to subspecies *fastidiosa*, sequence type 1, which has been reported to infect a variety of economically important crops, including cherry, plum, almond, and grapevine. A molecular clock analysis estimated that the introduction occurred between 2010 and 2020 (95% highest posterior density 1999 to 2022), with the strains forming a clade that diverged circa 2020. The findings underscore the importance of continued surveillance in both agricultural and unmanaged ecosystems, as the presence and potential impact of *X. fastidiosa* on Portuguese landscapes remain largely unknown.

Keywords: molecular clock analysis, phylogenetic analysis, plant pathogen surveillance, Portugal, whole-genome sequencing, *Xylella fastidiosa*

The worldwide spread of plant pathogens has led to numerous outbreaks with significant economic, environmental, and social impacts. According to estimates published by the Food and Agriculture Organization (FAO) in 2019, plant diseases result in an annual cost of $220 billion, with 20 to 40% of global agricultural production lost to pests (FAO 2019). Understanding the distribution, invasion routes, and reservoirs of these pathogens is crucial to anticipate, prevent, and mitigate emerging infectious diseases. In 2019, the European Commission published a list of 20 priority pests that pose the greatest economic, environmental, and social risks to European Union (EU) countries. Among them is the plant pathogen *Xylella fastidiosa* (European Commission 2019). A bacterium transmitted among plants by xylem sap-feeding insects, *X. fastidiosa* infects a wide range of plants, as evidenced by its detection from 712 plant species (EFSA et al. 2024). Originally from the Americas, *X. fastidiosa* was detected in Europe in 2013 in the Apulia region of Italy, where it caused substantial damage to olive trees, leading to a social and economic crisis (Saponari et al. 2019). Since then, it has been detected in other European countries, including France, Spain, and Portugal (Dupas et al. 2023; European Commission 2023; Román-Écija et al. 2023; Sicard et al. 2021). In Portugal, the first detection occurred in 2019, in one asymptomatic *Lavandula dentata* plant in the Porto region (EPPO 2019; Loureiro et al. 2023). Due to its high-risk status, *X. fastidiosa* is listed as a quarantine organism in the EU and is subject to strict regulations on handling, cultivation, and movement. These legal restrictions significantly limit the ability to grow and study the bacterium, making the isolation and genomic characterization of Portuguese strains particularly valuable.

X. fastidiosa comprises three distinct subspecies. *X. fastidiosa* subsp. *multiplex* is native to North America (Nunney et al. 2019) and has been introduced to Europe several times (Landa et al. 2020). It is known to infect almond, peach, plum, oak, and shade trees, among other plants (Landa et al. 2020; Trkulja et al. 2022). *X. fastidiosa* subsp. *fastidiosa*, native to Central America (Nunney et al. 2019), was introduced to the United States approximately 300 years ago (Donegan et al. 2024) and subsequently spread to Europe (Gomila et al. 2019), Taiwan (Castillo et al. 2019), and Israel (Zecharia et al. 2022). This subspecies is mostly associated with Pierce's disease (PD) of grapevines, which affects vineyards throughout the United States, leading to an estimated annual loss of $104.4 million in California alone (Tumber et al. 2014; Vanhove et al. 2020). Additionally, *X. fastidiosa* subsp. *fastidiosa* has been implicated in diseases affecting other plants, including almond, alfalfa, blueberry, and coffee (Davis et al. 1980; Oliver et al. 2015; Rodríguez et al. 2001). In Europe, it was first detected in cherry and myrtle leaf milkwort plants in Spain (Olmo et al. 2017) and later in grapevines (Gomila et al. 2019). *X. fastidiosa* subsp. *pauca* originates from South America, where it mainly infects orange, olive, and coffee (Coletta-Filho et al. 2016; de Lima et al. 1998; Rossetti et al. 1990). It was also present in Central America prior to its introduction to Europe (Nunney et al. 2012; Sicard et al. 2021) and has been identified in Costa Rica and the Apulian region of Italy (Giampetruzzi et al. 2017). Although the taxonomy of the species

†Corresponding author: J. Costa; jcosta@uc.pt

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is generally stable, recent reports of previously undescribed genetic diversity (Kahn and Almeida 2022) suggest that revisions may be needed due to phylogenetic uncertainties.

In addition to subspecies classification, *X. fastidiosa* has been grouped by sequence type (ST) based on multilocus sequence typing (MLST) of seven housekeeping genes (Scally et al. 2005). This tool provides cost-effective insights into genetic diversity without the often-challenging process of bacterial cultivation (EFSA et al. 2023). Such insights have been important for decision-makers in disease control strategies in Europe (EFSA et al. 2023). However, despite the usefulness of MLST in analyzing large numbers of samples, *X. fastidiosa* strains within the same ST can exhibit differences in host range (Nunney et al. 2019). Moreover, in some cases, the ST profile does not reconstruct the phylogeny inferred with whole-genome sequences (Landa et al. 2020), highlighting the limitations of MLST. Furthermore, it has become clear that genome sequences are required to reconstruct invasion pathways and to estimate when introductions occurred, which are key to the development of effective quarantine, eradication, and containment strategies.

Portugal is particularly vulnerable to *X. fastidiosa* due to its Mediterranean climate, the presence of insect vectors, and a diverse mosaic of potential host plants, including ornamental species, *Olea europaea*, *Vitis* spp., *Citrus* spp., and *Quercus* spp., which are widely distributed throughout the country (EFSA et al. 2019; Loureiro et al. 2023). In Portugal, the bacterium has been detected in suburban areas, scrublands, and forests, with no significant agricultural problems reported thus far. However, the number of detections is rising, now encompassing 19 officially demarcated outbreak areas, as defined by the National Phytosanitary Authority (DGAV), where the presence of *X. fastidiosa* has been confirmed by real-time PCR. These demarcated areas trigger mandatory surveillance and control measures following national and EU legislation, and the bacterium has been detected in 102 different plant species (DGAV 2024). This increase, coupled with the identification of both subspecies *fastidiosa* and *multiplex*, prompts concerns about its potential impact on agriculture and forests in the country. Despite the increasing number of cases and the associated economic and social risks, knowledge about *X. fastidiosa* in Portugal remains limited. Before this study, there were no *X. fastidiosa* isolates from Portugal, which limits the ability to predict the potential impact of the pathogen.

In 2022, *X. fastidiosa* was detected in the Beira Interior region of Portugal, the country's leading area for cherry and peach production, which includes two Protected Geographical Indications (Fundão and Cova da Beira) for these fruits (DGAV 2022). The region also has other *Prunus* species and a viticultural industry, both of which are potentially highly susceptible to *X. fastidiosa*. The region has a favorable climate for the development of *X. fastidiosa* diseases and an agroecosystem characterized by small agricultural plots surrounded by susceptible wild vegetation (such as *Adenocarpus* spp., *Cistus* spp., *Cytisus* spp., and *Ulex* spp.) (EFSA et al. 2024), which makes it especially vulnerable.

In this study, we present the genomic analysis of the first six *X. fastidiosa* strains isolated in Portugal, from the four outbreak-demarcated areas in the Beira Interior region. The genetic characterization aimed to determine their origin and time of introduction, providing crucial insights to help authorities implement control measures tailored to the local context.

Materials and Methods

Isolation of *X. fastidiosa* and DNA extraction

Sampling was conducted in October and November 2023 in four Portuguese *X. fastidiosa*-outbreak-demarcated areas defined by DGAV: Castelo Novo, Covilhã, Fundão, and Penamacor (DGAV 2023). Symptomatic and asymptomatic branches with leaves were collected from 28 plants (10 genera) for analysis (Supplementary Table S1). In the laboratory, segments of branches, petioles, and/or leaves (depending on the plant species) were selected from each sample and surface disinfected with a sequential treatment of 70% ethanol, followed by 1% sodium hypochlorite, and washed in distilled water, for 2 min each (adapted from Hill and Purcell 1995). For each sample, a portion of the branches and, when available, leaf petioles were gently compressed using pliers and directly blotted onto BCYE (buffered charcoal yeast extract agar) (Wells et al. 1981) and PD3 (Pierce's disease version 3) (Davis et al. 1981) culture media plates. Another portion of the selected plant segments from each sample was macerated in phosphate-buffered saline (PBS). Part of the resulting suspension was streaked onto the culture media plates, and the remainder was used for the detection of *X. fastidiosa* using the real-time PCR protocol described by Harper et al. (2010) (EPPO 2023). Plates were incubated for 3 to 4 weeks at 28°C, with regular monitoring of colony growth. Colonies with morphological characteristics resembling *X. fastidiosa* were subcultured on PD3 and BCYE media. Identification was performed using the previously mentioned real-time PCR protocol (Harper et al. 2010). The tests used to detect *X. fastidiosa* in plant extracts and in colonies were considered positive if they produced an exponential amplification slope and a Ct value below 38. Tests were considered negative if no amplification slope was observed or if the slope was non-exponential. Ct values above 38 were considered inconclusive. The *X. fastidiosa* isolates were cryopreserved at −80°C in a PD3 liquid medium with a final glycerol concentration of 30%.

Genomic DNA was purified from pure cultures according to the protocol of the MasterPure Complete DNA & RNA Purification Kit (LUCIGEN, Biosearch Technologies, Hoddesdon, U.K.). DNA quality and concentration were assessed using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, U.S.A.).

Sequence processing, genome assembly, and annotation

MinION whole-genome sequencing was performed by preparing a genomic library using the Native Barcoding Expansion with Ligation Sequencing Kit (EXP-NBD104 and SQK-LSK109, Oxford Nanopore Technologies [ONT], Oxford, U.K.) according to the manufacturer's protocol. The library was then loaded onto a flow cell (R9.4.1, FLO-MIN106) and sequenced for 10 h. MinKNOW software (v23.07.12) was then used to operate the MinION Mk1C (ONT), and basecalling was performed using Guppy (v7.14).

Long-read sequence quality control and genome assembly were performed using a combination of the web-based platforms Galaxy (Jalili et al. 2020) and NanoGalaxy (de Koning et al. 2020), following Sanders et al. (2021). Reads were filtered using Filtlong (v0.2.0) (https://github.com/rrwick/Filtlong) by removing sequences <1,000 bp, and quality was checked using NanoPlot (v1.28.2) (De Coster et al. 2018). De novo genome assembly was performed using Flye (v2.6) (Kolmogorov et al. 2019), and the results were evaluated using Bandage (Wick et al. 2015) and Quast (Gurevich et al. 2013) tools. After assembly, the resulting sequence was polished using Pilon (v1.20) (Walker et al. 2014), following Syme (2021).

Novogene CO, Ltd. was outsourced to perform short-read sequencing using Sequencer Illumina NovaSeq X Plus Series (PE150) and prepare the standard genomic library. The raw sequence data generated were processed for quality with FastQC (Andrews 2010), filtered with an average of 6,025,392 reads. Adapters were removed using Cutadapt (Martin 2011). Reads were trimmed using Seqtk (Shen et al. 2016) and then de novo assembled as contigs using SPAdes 3.12.0 on the *careful* setting (Bankevich et al. 2012). Subsequently, contigs were reordered using progressiveMauve (Darling et al. 2010) in comparison with the complete genome reference Temecula1, then annotated with Prokka (Seemann 2014). Hybrid assemblies of the Nanopore- and Illumina-generated reads were built using Unicycler (Wick et al. 2017) for all six strains.

The *X. fastidiosa* genomes were aligned with a set of 330 whole genomes publicly available of subsp. *fastidiosa* strains (Supplementary Table S2). The recently sequenced Herbarium sample Herb_1906, collected from *Vitis vinifera* in California in 1906, was included in the dataset to widen the sampling dates as much as possible for time calibration and ensure a measurably evolving population (Donegan et al. 2024; Drummond et al. 2003). These strains were aligned gene by gene using Panaroo (Tonkin-Hill et al. 2020) and the pipeline published in Donegan et al. (2024). The core genome was filtered for recombination using clonalframeML (Didelot and Wilson 2015), and then a maximum likelihood (ML) tree was built using RAxML (Stamatakis et al. 2008). The sequence type of the strains was determined using an MLST scheme based on seven housekeeping genes (Scally et al. 2005). These genes were extracted from each genome and analyzed through the PubMLST database (http://pubmlst.org). Basic diversity statistics were calculated using PopGenome (Pfeifer et al. 2014).

Inference of introduction date

To generate the time-calibrated phylogeny, Phylostems (Doizy et al. 2023) was used to confirm a significant temporal signal measured by linear regression of sample date to root-to-tip distances on the ML phylogeny. Single-nucleotide polymorphisms (SNPs) were extracted from the non-recombinant whole-genome alignment using SNP-sites (Page et al. 2016). Beast 1.10.5 (Suchard et al. 2018) was used to time calibrate the phylogeny using tip dating. A general time reversible substitution model was used to estimate substitutions, and an uncorrelated relaxed clock model with a log-normal distribution was used. The tree prior used was the coalescent Bayesian skyline with a random starting tree. One chain was run for 500 million steps, and parameters were logged every 50,000 steps. The first 50,000 steps were discarded as burn-in. Because of using only SNPs, the xml input file was manually edited to include the number of invariant G, A, T, and Cs. Convergence and sampling were confirmed with Tracer 1.7 (Rambaut et al. 2018) (ESS scores > 100), and TreeAnnotator (Drummond and Rambaut 2007) was used to determine the best-supported tree.

Results

X. fastidiosa detection and isolation

From the 27 plant samples, *X. fastidiosa* was detected in 6 plants, not detected in 17, and the results were inconclusive in 4 (Supplementary Table S1). Subsequently, *X. fastidiosa* was successfully isolated from all six qPCR-positive plants, with at least one isolate retrieved from each outbreak-demarcated area (Table 1). The locations of these plants are shown in Figure 1A and B, illustrating the distances among them. The two most distant plants, from which isolates 8188_Cistus_PT_2023 and 6273_Vitis_PT_2023 were obtained, were 45 km apart. In contrast, samples 8178_Cytisus_PT_2023 and 8179_Cytisus_PT_2023, both *Cytisus striatus*, were collected just a few meters apart. Whereas plant sample 8178_Cytisus_PT_2023 showed no symptoms, 8179_Cytisus_PT_2023 exhibited yellowing and had completely dry branches. The other samples were also symptomatic: 6273_Vitis_PT_2023 displayed symptoms consistent with PD (Fig. 1C), and 6250_Cytisus_PT_2023, 6258_Cytisus_PT_2023, and 8188_Cistus_PT_2023 had dry branches. Except for the *Vitis* sp. (which was growing by the side of a road, rather than in a commercial vineyard), all the plants from which *X. fastidiosa* was isolated were shrub and wild species typically found in Portuguese forest ecosystems.

Genome characteristics

The genome sequences of these six strains have been made publicly available (Table 2; associated with BioProject PRJNA1152915). The general features of the six genomes are detailed in Table 2. The genome sizes ranged from 2,542,905 to 2,564,847 bp, with the predicted number of protein-coding genes varying from 2,757 to 2,803. All strains had a GC content of 51.8%, except for 8179_Cytisus_PT_2023, which had 51.7%, and three genomes consisted of a single contig (6273_Vitis_PT_2023, 8178_Cytisus_PT_2023, and 8188_Cistus_PT_2023), whereas the other three contained 26, 27, and 29 contigs (6250_Cytisus_PT_2023, 6258_Cytisus_PT_2023, and 8179_Cytisus_PT_2023), respectively. The *X. fastidiosa* ST of all sequenced strains was ST1. In an alignment of the hybrid assemblies of these six strains alone, there were 334 segregating sites, nucleotide diversity (Pi) was 6.36e-05, Tajima's D was −0.77, and Waterson's theta was 7.22e-05. These values suggest a recent bottleneck in the population, in this case interpreted as a recent introduction.

Phylogenetic inferences

The time-calibrated phylogenetic tree (Fig. 2) showed that the six strains formed a single clade with a posterior probability of 1 (the highest possible). This clade, introduced in Portugal, is nested within a larger clade of strains isolated from Temecula in Southern California in 2016, as well as one strain isolated from Napa Valley in 2011. There was a significant temporal signal in the data at the node ancestral to all North American strains (including those associated with subsequent introductions into Spain, Israel, and Portugal) with a P value of 0.0233, an R^2 of 0.013, and a mean rate of 2.1276×10^{-6} (95% highest posterior density [HPD] 8.9371×10^{-7} to 4.8072×10^{-6}).

Using the median heights and 95% HPD intervals, the ancestral node of the Portuguese strains is estimated to date back to approximately 2020 (95% confidence interval [CI] 2019 to 2022). However, the common ancestor node of these six strains and the group from California is estimated to date back to 2010 (95% CI 1999 to 2016). The introduction could have occurred at any point along this branch. It is plausible that, due to sampling limitations, the six strains analyzed may not represent the full genetic diversity of the introduction but rather a subset of the diversification that has occurred since. The gap between these two dates (2010 and 2020) likely represents unsampled genetic biodiversity, either still present in California or within the Portuguese population, which remains to be sampled.

Discussion

The six strains analyzed in this study belong to a clade of PD-causing *X. fastidiosa* subsp. *fastidiosa*, a lineage originating in Central America that was introduced into the United States approximately 300 years ago (Donegan et al. 2024). All strains belong to ST1, reported to infect 66 different plant species (EFSA et al. 2024), including economically important crops present in the Beira Interior

TABLE 1. *Xylella fastidiosa* strains isolated in Portugal, including their host plant species, geographical region, and genome accession numbers

X. fastidiosa strain	Host plant species	Demarcated area	Genome accession number
6250_Cytisus_PT_2023	*Cytisus* sp.	Fundão	SAMN43379328
6258_Cytisus_PT_2023	*Cytisus* sp.	Fundão	SAMN43379329
6273_Vitis_PT_2023	*Vitis vinifera*	Castelo Novo	SAMN43379330
8178_Cytisus_PT_2023	*Cytisus striatus*	Covilhã	SAMN43379331
8179_Cytisus_PT_2023	*Cytisus striatus*	Covilhã	SAMN43379332
8188_Cistus_PT_2023	*Cistus ocymoides*	Penamacor	SAMN43379333

region of Portugal, such as *Prunus* spp. and *Vitis vinifera*. Additionally, plant species commonly found in forested areas, such as *Acacia dealbata*, *Cistus* spp., and *Cytisus striatus*, are also included in this list, suggesting that they may serve as potential reservoirs for the bacterium. Except for the European grapevine (*Vitis vinifera*; 6273_Vitis_PT_2023), all other host species from which isolates were obtained are native Portuguese plants. However, the *V. vinifera* specimen was located along a roadside, rather than in a commercial vineyard. This observation aligns with the hypothesis that these plants could act as reservoirs for agricultural species, as illustrated by the isolation of one strain (6258_Cytisus_PT_2023) near the edge of a *P. persica* orchard.

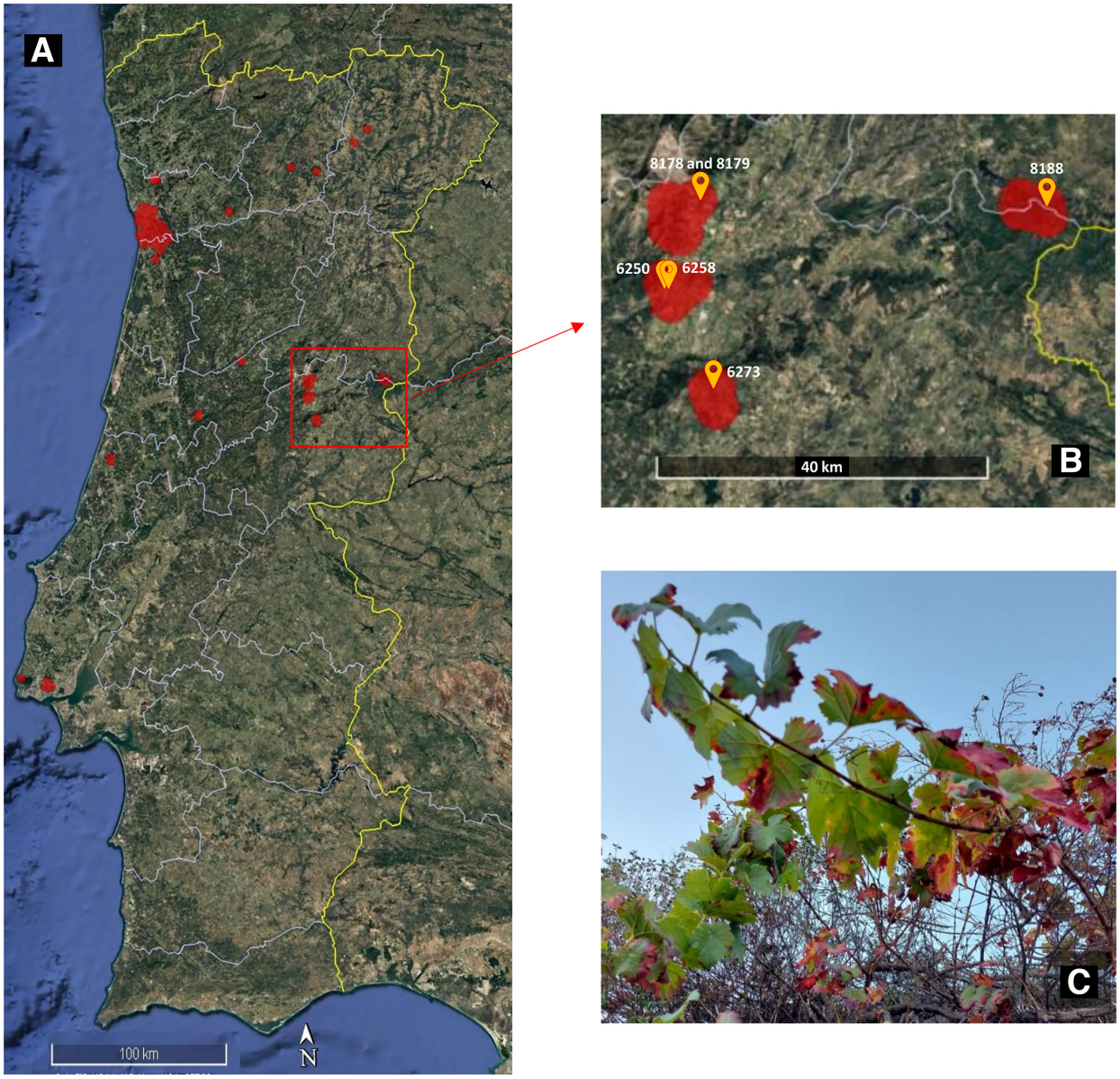

Fig. 1. A, Demarcated areas of *Xylella fastidiosa* in Portugal in 2023 are highlighted in red. **B,** Demarcated areas of the Beira Interior region, with yellow pins indicating the locations of plants from which *X. fastidiosa* was isolated. **C,** Grapevine plant showing symptoms similar to Pierce's disease, from which isolate 6273_Vitis_PT_2023 was obtained.

TABLE 2. General features of the whole-genome sequences of the *Xylella fastidiosa* strains isolated in Portugal

Features	6250_Cytisus_ PT_2023	6258_Cytisus_ PT_2023	6273_Vitis_ PT_2023	8178_Cytisus_ PT_2023	8179_Cytisus_ PT_2023	8188_Cistus_ PT_2023
Genome size (bp)	2,564,847	2,548,784	2,542,905	2,551,517	2,547,088	2,550,222
GC content (%)	51.8	51.8	51.8	51.8	51.7	51.8
Number of contigs	26	27	1	1	29	1
N50	2,507,618	2,485,397	–	–	2,081,468	–
Predicted coding sequences	2,803	2,765	2,757	2,786	2,777	2,782
Coverage	356×	238×	291×	294×	286×	294×
Number of rRNAs	56	56	56	56	56	56
tRNA	41	41	41	41	41	41
Coding sequences	1,234	1,226	1,225	1,230	1,233	1,234
rRNA	4	4	4	4	4	4

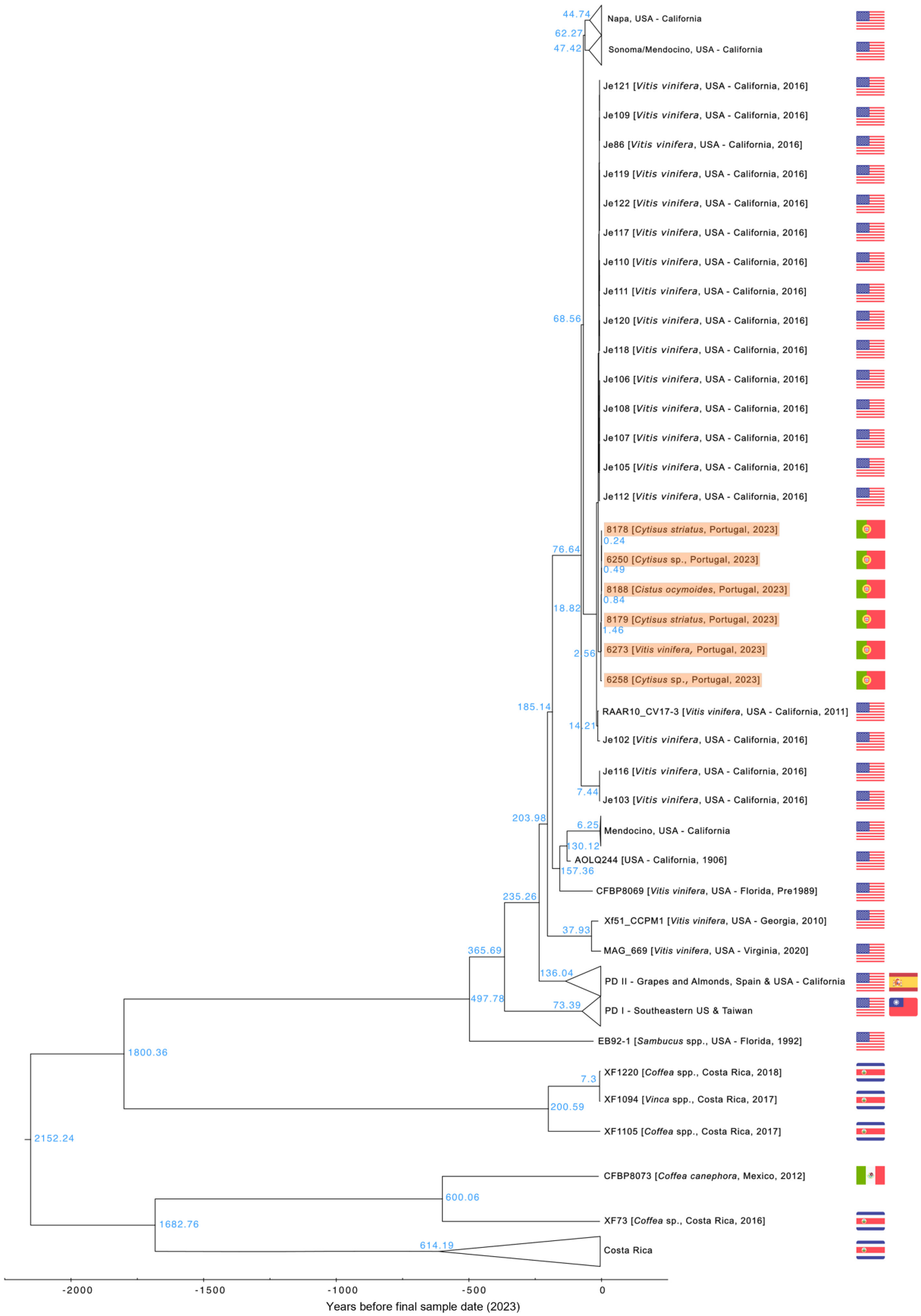

Fig. 2. Time-calibrated phylogenetic tree based on the genome of selected *Xylella fastidiosa* subsp. *fastidiosa* strains. Strains sequenced in this study are highlighted with orange boxes. For each strain, the country of origin, host plant species, and date of isolation are given in brackets. Clustered strains are detailed in Supplementary Table S2.

The data indicate that these *X. fastidiosa* outbreaks in the Beira Interior region of Portugal resulted from a single introduction event from California. Although there are several introductions of *X. fastidiosa* into European countries, such as Spain, France, and Italy (Dupas et al. 2023; Román-Écija et al. 2023; Sicard et al. 2021), there is no evidence linking these outbreaks to the Portuguese strains studied here, indicating that they likely have independent origins.

Diversity statistics from our study, based on the alignment of the hybrid assemblies of these six strains, are comparable to those reported by Zecharia et al. (2022), who conducted similar analyses on related populations of *X. fastidiosa* subsp. *fastidiosa* also connected to a single recent introduction; their findings indicate similar levels of nucleotide diversity and selection in recent outbreaks of the subspecies in Taiwan, Israel, and Spain. Our results, with a Tajima's D of −0.77, also suggest that the introduction of *X. fastidiosa* into the Beira Interior region is recent.

Molecular time inferences revealed two significant time estimates. The first is the estimated age of the clade containing the six strains dates to approximately 2.5 years before the collection of the oldest sample, placing its origin around 2020 (95% CI 2019 to 2022). The second relevant date is the estimated time at which this clade diverged from the California lineage, which is approximately 2010 (95% CI 1999 to 2016). This suggests a temporal gap of between 3 and 23 years during which the evolutionary and geographical trajectory of these strains remains unresolved. One possibility is that the strains underwent further diversification in California or at an intermediate location before their introduction into Portugal, reflecting the potential for one or more undetected transmission events. Alternatively, there may be greater genetic diversity of *X. fastidiosa* in Portugal than was captured in the present study. Comprehensive sampling across additional geographical areas and host plants is required to determine whether the introduction was direct from California or via an intermediate location and to identify any unrecognized diversity within the region.

It is important to note that this study covers only four of the 19 *X. fastidiosa* demarcated areas in Portugal (DGAV 2024). Currently, it is known that two subspecies occur in Portugal: *X. fastidiosa* subsp. *fastidiosa* (in the Alentejo, Centre, Lisboa e Vale do Tejo, and North regions) and subsp. *multiplex* (in the North, Centre, and Lisboa e Vale do Tejo regions) (DGAV 2024). Furthermore, in addition to ST1, ST2 has been identified in a plant in the Lisboa e Vale do Tejo region (DGAV 2024). The lack of isolates limits our understanding of the bacterium's genetic diversity, potential host plants, and patterns of dispersion into and within Portugal. These gaps are further compounded by the quarantine status of *X. fastidiosa* in the EU, which imposes strict restrictions on the isolation, cultivation, and experimental use of live strains. As a result, very few laboratories in Portugal are authorized to handle this pathogen, making field-based and genomic studies particularly challenging. This study helps address that gap by providing the first cultured isolates and genomic data for the country.

The increasing number of demarcated areas in which the bacterium has been detected, the proximity of agricultural importance regions, and the diversity of *X. fastidiosa* identified so far emphasize the urgent need to intensify sampling efforts in crop areas. The potential presence and impact of *X. fastidiosa* on local agricultural production remain largely unknown and require investigation to effectively anticipate, prevent, and mitigate the consequences of its spread.

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