**Article**

**Use of traC Gene to Type the Incidence and Distribution of pXFAS_5235 Plasmid-Bearing Strains of Xylella fastidiosa subsp. fastidiosa ST1 in Spain**

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**Abstract:** Xylella fastidiosa (Xf) is a phytopathogenic bacterium with a repertoire of self-replicating genetic elements, including plasmids, pathogenicity islands, and prophages. These elements provide potential avenues for horizontal gene transfer both within and between species and have the ability to confer new virulence traits, including the ability to colonize new host plants. However, they can also serve as a ‘footprint’ to type plasmid-bearing strains. Genome sequencing of several strains of Xf subsp. fastidiosa sequence type (ST) 1 from Mallorca Island, Spain, revealed the presence of a 38 kb plasmid (pXFAS_5235). In this study, we developed a PCR-based typing approach using primers targeting the traC gene to determine the presence of pXFAS_5235 plasmid or other plasmids carrying this gene in a world-wide collection of 65 strains X. fastidiosa from different subspecies and STs or in 226 plant samples naturally infected by the bacterium obtained from the different outbreaks of Xf in Spain. The traC gene was amplified only in the plant samples obtained from Mallorca Island infected by Xf subsp. fastidiosa ST1 and from all Spanish strains belonging to this ST. Maximum-likelihood phylogenetic tree of traC revealed a close relatedness among Spanish and Californian strains carrying similar plasmids. Our results confirm previous studies, which suggested that a single introduction event of Xf subsp. fastidiosa ST1 occurred in the Balearic Islands. Further studies on the presence and role of plasmids in Xf strains belonging to the same or different subspecies and STs can provide important information in studies of epidemiology, ecology, and evolution of this plant pathogen.

**Keywords:** phylogenetic analysis; plasmids; quarantine; traC gene; Xylella fastidiosa

1. **Introduction**

Xylella fastidiosa (Xf) is a xylem-inhabiting plant pathogenic bacterium, native from the Americas, which can infect more than 650 plant species that have been reported as hosts [1]. This bacterium causes a variety of diseases on crops of high economic importance, including grapevine (Pierce’s disease, PD), citrus (Citrus Variegated Chlorosis, CVC), coffee (Coffee Leaf Scorch,CLS), almond trees (Almond Leaf Scorch, ALS), and olive trees (Olive Quick Decline Syndrome, OQDS) [1]. Currently, three Xf subspecies have been officially described (fastidiosa, multiplex, and pauca) [2–4] and two additional ones have been proposed (morus and sandvi) [5,6], but based on genomic analyses are included in the subspecies fastidiosa [7]. To date, within the subspecies level, 89 sequence types (ST) have been described based on MultiLocus Sequence Typing analysis (MLST) of seven housekeeping genes (cysG, gltT, hoIC, leuA, malF, nuoL, and petC) (https://pubmlst.org/organisms/xylella-fastidiosa; accessed on 12 August 2022) [8,9].
In Europe, the detection of Xf in the Salento region in Italy in 2013, which is associated with a massive mortality of olive trees [10,11] heightened big concerns on the risk of its spread across the continent. As a result, mandatory surveys of EU member states [12,13] were conducted revealing that the bacterium was also established in Corsica and Provence-Alpes-Côte d’Azur in France in 2015 [14], the Balearic Islands and Alicante in Spain in 2016 and 2017 [15,16], and in Porto, Lisbon, and Algarve in Portugal in 2019 and 2021 [17].

Results of official surveys in Balearic Islands since 2016 have revealed that the Xf genetic diversity in the islands is the highest within the different outbreaks in Europe. Therefore, to date, three Xf subspecies and five STs have been detected in the Balearic Islands [18–20]: (i) Xf subsp. fastidiosa ST1, which causes PD and ALS in California and also in Spain, recorded only on Mallorca Island; (ii) a new ST of Xf subsp. pauca (ST80), which is found only on Ibiza Island, mainly on wild and cultivated olive trees (Olea europaea); and (iii) another novel ST of Xf subsp. multiplex (ST81), which is present in the Mallorca and Menorca islands affecting mainly almonds, cultivated and wild olives, and Ficus carica. In addition, there have been three single detections of Xf subsp. multiplex ST7 in Mallorca Island on Prunus dulcis and Polygala myrtifolia.

In a recent study, phylogenetic analysis based on core genomes showed that isolates from Xf subsp. fastidiosa ST1 and Xf subsp. multiplex ST81 in the Balearic Islands shared their most recent common ancestors with Californian Xf populations. These isolates from California were associated with PD and ALS in grapevines and almonds, respectively, [18,20]. Moreover, all of the genomes obtained from several strains of Xf subsp. fastidiosa ST1 isolated from grapes and almonds in Mallorca revealed the presence of a 38 kb plasmid (pXFAS_5235), with a high sequence similarity to the conjugative plasmid pXFAS01 from isolate M23 of the same subspecies and ST, causing ALSD in California [18,21,22]. Interestingly, a nearly identical plasmid was found in Xf subsp. multiplex strain RIV5 [23]. The occurrence of similar plasmids in different subspecies of Xf represents an evidence of horizontal gene transfer (HGT) between strains [23] and together with recombination are hypothesized as one of the forces in the emergence of new Xf strains, which are capable of colonizing novel hosts [24].

It is known that Xf possesses a repertoire of mobile genetic elements (MGE), including prophages, pathogenicity islands, and plasmids [24]. The presence of large indigenous plasmids is frequent in bacteria that interact with plants, and in some circumstances, they confer virulence traits to plant-pathogenic bacteria, such as the Ti plasmid of Agrobacterium tumefaciens [25]. Bacterial plasmids are extrachromosomal DNA sequences, which are capable of autonomous replication and are a major source of HGT among bacteria, contributing to their evolution and adaptation [24,26]. Plasmids can be classified as self-transmissible or conjugative if they code for their own set of mating pair formation (MPF) proteins, such as the type IV coupling proteins (T4CP) and VirB4, which are part of the type IV secretion system (T4SS), enabling bacteria to efficiently exchange genetic material and proteins [27]. Therefore, plasmids play a very important role in bacterial pathogenesis and evolution.

Plasmid comparative sequence analyses and studies of their presence and distribution among different Xf strains belonging to the same or different subspecies and STs can provide important information regarding adaptation mechanisms of Xf strains to their hosts, with application in future studies of epidemiology, ecology, and evolution of this plant pathogen. Therefore, in this study, we used a PCR-based approach to describe the incidence and distribution of the pXFAS_5235 plasmid on a collection of several Xf strains from different subspecies and STs isolated from different host plants, different regions worldwide, and DNA samples extracted from Xf-infected plant samples, which are obtained from several hosts from different outbreak areas in Spain. On the basis of the hypothesis that the introduction of Xf in the Balearic Islands may have occurred in the early 1990s with the import of almond varieties [18], the analysis of the presence of pXFAS_5235 plasmid on Xf Spanish populations may provide interesting epidemiological information concerning the existence of different introduction events or of potential adaptation of the strains after some decades, since their introduction or after hosts jump from almonds into
other crops and plants from the natural environment [19]. The objectives of this study were: (i) To develop a PCR-based typing approach using primers targeting the \textit{traC} gene to determine the presence of pXFAS\_5235 plasmid or other plasmids carrying this gene in a world-wide collection of \textit{X. fastidiosa} strains from different subspecies and STs. (ii) To use this PCR-based typing approach for testing the presence of pXFAS\_5235 plasmid in plant samples naturally infected by the bacterium obtained from the different outbreaks of \textit{Xf} in Spain. (iii) To support the hypothesis that a single introduction event of \textit{Xf} subsp. \textit{fastidiosa} ST1 occurred in the Balearic Islands.

2. Results

2.1. Sensitivity of the \textit{traC} PCR-Based Plasmid Typing Protocol

The \textit{traC} gene involved in DNA replication and transcription (locus AGC23499; previously D698\_p2018) [23] was selected to type the presence of pXFAS\_5235 plasmid. The sensitivity of the primer pair was evaluated on serial dilutions of \textit{Xf} subspecies \textit{fastidiosa} strain IVIA5235 DNA in different plant DNA backgrounds calibrated at an initial concentration of $5 \times 10^5$ copies/µL. The latest and reproducible PCR amplification signal was obtained at 500 copies/µL and for all plant DNA backgrounds. However, for some of the two replications that were assessed, a very weak amplification could be observed at 50 copies/µL. However, it was not reproducible among the different experiments using independent standard curves and PCR replications, especially when using \textit{Polygala} and insect (\textit{N. campestris}) DNA backgrounds (Figure 1A).

In addition, the same standard curves were assessed by qPCR using Harper’s test for comparison with the detection limit of the newly-developed \textit{traC} typing PCR. The Ct values for Harper’s qPCR that corresponded to 500 copies/µL ranged between 27.7 and 29.7 and those corresponding to 50 copies/µL ranged between 31.8 and 33.5, depending on the DNA background (host plant and insect DNA) and replication; however, the results were very reproducible (Figure 1B). These results indicated that for those plant DNA samples, which are naturally infected by \textit{Xf} showing Harper’s qPCR Ct values higher or close to 28, there may be some chance of getting no amplification of the \textit{traC} gene using the ND116\_pRIV5\_F1/ND117\_pRIV5\_R1 primer pair, even if the \textit{Xf} strain infecting the sample may harbor the pXFAS\_5235 plasmid (Figure 1A).

2.2. PCR-Based Plasmid Typing of \textit{Xylella fastidiosa} Strains and Naturally-Infected Plant Samples

A total of five strains isolated in Mallorca Island belonging to \textit{Xf} subsp. \textit{fastidiosa} ST1 amplified the \textit{traC} gene present in the pXFAS\_5235 plasmid. The remaining Spanish strains analyzed belonging to \textit{Xf} subsp. \textit{multiplex} ST6 and ST81 or to \textit{Xf} subsp. \textit{pauca} ST80 showed no amplification of the \textit{traC} gene (Table 1; Figure S1). Moreover, amplification was obtained from other strains of \textit{Xf} subsp. \textit{fastidiosa} ST1 (Temecula1, Temecula, M23, and CFBP8351), and two \textit{Xf} subsp. \textit{fastidiosa} ST2 strains (CFBP7970 and CFBP8082). \textit{Xf} subsp. \textit{multiplex} ST6 (strain Dixon) was the only strain from those tested belonging to subspecies \textit{multiplex}, in which the \textit{traC} gene was amplified. Furthermore, several \textit{Xf} subsp. \textit{pauca} strains from ST53 (CODIRO, De Donno, Salento-1, CFBP8495, and CFBP8429), ST73 (CFBP8498) and ST74 (CFBP8072 and CFBP8074), the recombinant strain CO33 of subsp. \textit{sandyi/morus}, and strain CFBP8478 of \textit{Xf} subsp. \textit{sandyi} amplified the \textit{traC} gene (Table 1; Figure S1).

A total of 47 from 52 naturally-infected plant samples from Mallorca Island, which were previously characterized as infected by \textit{Xf} subsp. \textit{fastidiosa} ST1 with MLST analysis amplified the \textit{traC} gene, indicating the presence of pXFAS\_5235 plasmid. On the other hand, we could not amplify the \textit{traC} gene in the remaining five plant samples. These samples showed a Ct $> 30$ for Harper’s qPCR test, which was over the detection limit of the ND116\_pRIV5\_F1 and ND117\_pRIV5\_R1 primer pair. Indeed, for those samples, it was necessary to perform the Nested-MLST analysis [28] to assign the \textit{Xf} isolate infecting the sample at the subspecies and ST level.
Figure 1. (A) Agarose gels showing the sensitivity of ND116-pRIV5-F1/ND117-pRIV5-R1 primer pair in the amplification of the traC gene (expected amplicon size of 521 bp) using serial dilutions of Xylella fastidiosa (Xf) subspecies fastidiosa strain IVIA5235 DNA (from $5 \times 10^5$ copies/µL; lanes 2 to 8) singly or mixed with host DNA (almond, grapevine, olive, polygala, Neophilaenus campestris, and water). NIC: Negative isolation control included plant insect DNA only. PAC: Positive amplification control contained boiled cells of IVIA5235 strain. NAC: Negative amplification control contained DNA from a Xf strain known not to harbor the traC gene. L: 10 kb GeneRuler™ DNA Ladder Mix (Thermo Scientific™). Data shown below agarose gels summarize the results from two independent standard DNA curves (1 and 2): +L: Positive amplification; ±: Positive weak amplification; and −: No amplification. (B) Cycle thresholds obtained for Harper’s qPCR using the same DNA standard curves. Data correspond to three PCR amplifications from the serial DNA standard curve number 1, shown in (A).
Table 1. *Xylella fastidiosa* strain collection at the Institute of Sustainable Agriculture, Córdoba, Spain (IAS-CSIC), used in the study, which include different subspecies and sequence types (ST) and the results of the PCR-based typing of *traC* gene using the ND116-pRIV5-F1/ND117-pRIV5-R1 primer pair.

| Subspecies a | ST a | Strain | Origin | Host | traC Gene b |
|--------------|------|--------|--------|------|-------------|
| *fastidiosa* 1 | IVIA5235 | Balearic Island, Spain | *Prunus avium* | + |
|               | IVIA5770 | Balearic Island, Spain | *Vitis vinifera* | + |
|               | R2XF4358/18 | Balearic Island, Spain | *Rhamnus alaternus* | + |
|               | XYL3349 | Balearic Island, Spain | *Prunus dulcis* | + |
|               | CFBP8351 | California, USA | *Vitis vinifera* | + |
|               | Temecula1 | California, USA | *Vitis vinifera* | + |
|               | M23 | California, USA | *Prunus dulcis* | + |
| *fastidiosa* 2 | CFBP9790 | Florida, USA | *Vitis vinifera* | + |
|               | CFBP8082 | Florida, USA | *Ambrosia artemifolia* | + |
|               | WM1-1 | Georgia, USA | *Vitis vinifera* | – |
|               | CFBP7969 | North Carolina, USA | *Vitis rotundifolia* | – |
|               | CFBP8083 | North Carolina, USA | *Vitis vinifera* | – |
| *fastidiosa* 75 | CFBP8073 | Mexico | *Coffea canephora* | – |
| *morus* 29 | CFBP8084 | Georgia, USA | *Morus alba* | – |
| *multiplex* 6 | Dixon | California, USA | *Prunus dulcis* | + |
|               | ESL | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IVIA6902 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX212H7 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX235T1 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX235T10 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX64H11 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX64T12 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX64T13 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS-AFX64T14 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IAS9001 | Valencian Community, Spain | *Prunus dulcis* | – |
|               | IVIA6586-2 | Valencian Community, Spain | *Helicrysum italicum* | – |
|               | IVIA6629 | Valencian Community, Spain | *Rhamnus alaternus* | – |
|               | CFBP8417 | Corsica, France | *Spartium junceum* | – |
|               | CFBP8418 | Corsica, France | *Spartium junceum* | – |
| *multiplex* 7 | CFBP8416 | Corsica, France | *Polygon lycium* | – |
|               | M12 | California, USA | *Prunus dulcis* | – |
|               | LM10 | California, USA | *Olea europaea* | – |
|               | RH1 | California, USA | *Olea europaea* | – |
| *multiplex* 10 | CFBP8070 | Georgia, USA | *Prunus sp.* | – |
| *multiplex* 27 | CFBP8075 | California, USA | *Prunus sp.* | – |
| *multiplex* 41 | CFBP8173 | Georgia, USA | *Prunus salicina* | – |
|               | CFBP8068 | Washington DC, USA | *Ulmus sp.* | – |
| *multiplex* 42 | AlmaEm3 | Georgia, USA | *Vaccinium sp.* | – |
| *multiplex* 43 | BB08-1 | Florida, USA | *Vaccinium corymbosum* | – |
| *multiplex* 51 | CFBP8078 | Florida, USA | *Vinca sp.* | – |
| *multiplex* 81 | XYL1981/17 | Balearic Islands, Spain | *Ficus carica* | – |
|               | XYL1966/18 | Balearic Islands, Spain | *Olea europaea* | – |
|               | XYL468 | Balearic Islands, Spain | *Olea europaea* | – |
|               | XYL466/19 | Balearic Islands, Spain | *Olea europaea* | – |
|               | XF3348 | Balearic Islands, Spain | *Prunus dulcis* | – |
|               | XYL1752/17 | Balearic Islands, Spain | *Prunus dulcis* | – |
|               | Santa29b | Balearic Islands, Spain | *Santolina chamaecyparissus* | – |
|               | Fillmore | California, USA | *Olea europaea* | – |
Table 1. Cont.

| Subspecies a | ST a | Strain | Origin | Host | traC Gene b |
|--------------|------|--------|--------|------|-------------|
| *pauca*      | 53   | DeDonno | Apulia, Italy | *Olea europaea* | + |
|              |      | CFBP8477/Salento-1 | Apulia, Italy | *Olea europaea* | + |
|              |      | CFBP8402/CoDiRo | Apulia, Italy | *Olea europaea* | + |
|              |      | CFBP8495/PD7202 | Intercepted, Costa Rica c | *Coffea arabica* | + |
|              |      | CFBP8429 | Intercepted, unknown c | *Coffea arabica* | + |
| *pauca*      | 73   | CFBP8498/PD7211 | Intercepted, Costa Rica c | *Coffea arabica* | + |
| *pauca*      | 74   | CFBP8072 | Ecuador | *Coffea arabica* | + |
|              |      | CFBP8074 | Ecuador | *Coffea arabica* | + |
| *pauca*      | 80   | XYL1961 | Balearic Island, Spain | *Olea europaea* | – |
|              |      | IAS-XYL1513-1 | Balearic Island, Spain | *Prunus dulcis* | – |
|              |      | IAS-XYL1518 | Balearic Island, Spain | *Prunus dulcis* | – |
| *sandyi*    | 5    | Ann-1 | California, USA | *Nerium oleander* | – |
| *sandyi*    | 72   | CFBP8478 | Intercepted, Costa Rica c | *Coffea arabica* | + |
| *sandyi*    | 76   | CFBP8356 | Intercepted, Costa Rica c | *Coffea arabica* | – |
| *sandyi/morus* | 72  | CO33 | Intercepted, Costa Rica c | *Coffea arabica* | + |

Subspecies and sequence type (ST) were determined by MLST analysis or by BLAST search of whole genome against the *Xylella fastidiosa* MLST database (https://pubmlst.org/xfastidiosa/). Presence of *traC* gene by PCR-based plasmid typing was performed using ND116-pRIV5-F1 and ND117-pRIV5-R1 primers, pairs developed in this study. Positive and negative amplifications are represented as + or −, respectively. Strains were isolated on the countries indicated from *Xf*-positive intercepted plants. Strains CFBP8495 and CFBP8498 were intercepted in Netherlands from plants from Costa Rica; CO33 was intercepted in Italy from plants from Costa Rica. Strains CFBP8478 and CFBP8356 were intercepted in France from plants from Costa Rica, and CFBP8429 were intercepted in France from plants of unknown origin.

Nine of the 23 plant samples obtained from almond trunk rings, and previously shown as infected by *Xf* subsp. *fastidiosa* amplified the *traC* gene, indicating the presence of pXFAS_5235 plasmid. From these positive samples, the DNA tree ring sample dated before 2004 was the oldest showing amplification (Table 2). For the remaining *Xf* subsp. *fastidiosa*-infected samples, in which there was no amplification of the *traC* gene, the Ct values shown in Harper’s qPCR ranged between 25 and 36.

Table 2. Plant samples used in the study naturally infected by *X. fastidiosa*, with the information of host, geographical origin, and results of amplification of *traC* gene, which was used as an indicator of the presence of pXFAS_5235 plasmid.

| Subspecies a | ST a | Origin | Host b | Ct c | Number of Samples d | traC Gene e |
|--------------|------|--------|--------|------|---------------------|-------------|
| *fastidiosa* 1 | Mallorca | Calicotome spinosa | 23, 27 | 2 | 2 |
| *fastidiosa* 1 | Mallorca | Genista lucida | 20 | 1 | 1 |
| *fastidiosa* 1 | Mallorca | Juglans regia | 29 | 1 | 1 |
| *fastidiosa* 1 | Mallorca | Polysuga myrtifolia | 23–28 | 4 | 4 |
| *fastidiosa* 1 | Mallorca | Prunus dulcis | 21–31 | 23 | 19 |
| *fastidiosa* 1 | Mallorca | Prunus dulcis TR < 2004 | 25 | 2 | 2 |
| *fastidiosa* 1 | Mallorca | Prunus dulcis TR 2005–2009 | 22 | 2 | 2 |
| *fastidiosa* 1 | Mallorca | Prunus dulcis TR 2010–2017 | 22 | 5 | 5 |
| *fastidiosa* 1 | Mallorca | Prunus dulcis TR 1996–2015 | 25–26 | 14 | 0 |
| *fastidiosa* 1 | Mallorca | Rhamnus alaternus | 20–27 | 8 | 8 |
| *fastidiosa* 1 | Mallorca | Ruta chalepensis | 17–27 | 3 | 3 |
| *fastidiosa* 1 | Mallorca | Teucrium capitatum | 33 | 1 | 0 |
| *fastidiosa* 1 | Mallorca | Vitis vinifera | 21–25 | 9 | 9 |
| *multiplex* 6 | Alicante | Prunus dulcis | 18–29 | 22 | 0 |
| *multiplex* 7 | Mallorca | Polysuga myrtifolia | 28 | 1 | 0 |
| *multiplex* 7 | Mallorca | Prunus dulcis | 24, np | 2 | 0 |
Table 2. Cont.

| Subspecies | ST | Origin | Host   | Ct | Number of Samples | traC Gene |
|------------|----|--------|--------|----|------------------|-----------|
| multiplex  | 81 | Mallorca | Acacia saligna | 29 | 1 | 0 |
| multiplex  | 81 | Mallorca | Calicotome spinosa | 25 | 1 | 0 |
| multiplex  | 81 | Mallorca | Cistus albidus | 33, 34 | 2 | 0 |
| multiplex  | 81 | Mallorca | Ficus carica | 26–32 | 4 | 0 |
| multiplex  | 81 | Mallorca | Fraxinus angustifolia | 24–30 | 9 | 0 |
| multiplex  | 81 | Mallorca | Genista valdes-bermejoi | 24 | 1 | 0 |
| multiplex  | 81 | Mallorca | Helichrysum stoechas | 28 | 1 | 0 |
| multiplex  | 81 | Mallorca | Lavandula dentata | 25, 28 | 2 | 0 |
| multiplex  | 81 | Mallorca | Olea europaea | 20–33 | 22 | 0 |
| multiplex  | 81 | Mallorca | Prunus dulcis | 25, 26 | 2 | 0 |
| multiplex  | 81 | Mallorca | Rhamnus alaternus | 27 | 2 | 0 |
| multiplex  | 81 | Mallorca | Rosmarinus officinalis | 33 | 1 | 0 |
| multiplex  | 81 | Mallorca | Salvia officinalis | 23 | 1 | 0 |
| multiplex  | 81 | Mallorca | Santolina chamaecyparissus | 20, 22 | 2 | 0 |
| multiplex  | 81 | Mallorca | Spartium junceum | 21, 24 | 2 | 0 |
| multiplex  | 81 | Menorca | Cistus albidus | 25, 26 | 2 | 0 |
| multiplex  | 81 | Menorca | Clematis cirrhosa | 30 | 1 | 0 |
| multiplex  | 81 | Menorca | Ficus carica | 28 | 1 | 0 |
| multiplex  | 81 | Menorca | Helichrysum stoechas | 21, 27 | 2 | 0 |
| multiplex  | 81 | Menorca | Olea europaea | 24–30 | 11 | 0 |
| multiplex  | 81 | Menorca | Phlomis italica | 25, 27 | 2 | 0 |
| multiplex  | 81 | Menorca | Rhamnus alaternus | 24, 35 | 2 | 0 |
| multiplex  | 81 | Menorca | Rosmarinus officinalis | 26, 29 | 2 | 0 |
| multiplex  | 81 | Menorca | Santolina chamaecyparissus | 24–31 | 5 | 0 |
| multiplex  | 81 | Menorca | Santolina magonica | 29 | 1 | 0 |
| multiplex  | 81 | Menorca | Vitex agnus-castus | 31 | 1 | 0 |
| pauca      | 80 | Ibiza   | Acacia saligna | 23, 24 | 2 | 0 |
| pauca      | 80 | Ibiza   | Cistus albidus | 25–30 | 3 | 0 |
| pauca      | 80 | Ibiza   | Genista hirsuta | 22 | 2 | 0 |
| pauca      | 80 | Ibiza   | Lavandula angustifolia | 23 | 1 | 0 |
| pauca      | 80 | Ibiza   | Lavandula dentata | 29 | 1 | 0 |
| pauca      | 80 | Ibiza   | Olea europaea | 24–32 | 15 | 0 |
| pauca      | 80 | Ibiza   | Polygala myrtifolia | 28, 31 | 2 | 0 |
| pauca      | 80 | Ibiza   | Prunus dulcis | 29–33 | 9 | 0 |
| pauca      | 80 | Ibiza   | Rosmarinus officinalis | 29, np | 3 | 0 |
| pauca      | 80 | Ibiza   | Ulex parviflorus | 21–25 | 2 | 0 |

a Subspecies and sequence type (ST) were determined by MLST [8] or Nested-MLST [28] analysis. b All of the samples were obtained from fresh leaf petioles or stem portions of all analyzed hosts; except for samples of Prunus dulcis obtained from tree rings (TR) from a previous study [20]. c Ct: Cycle threshold value obtained for Xf amplification on those samples using Harper’s qPCR test [29]. Np: Not performed. d Number of samples analyzed for each host plant sampled during different years (2018–2022) and locations in official surveys. e Number of samples showing a positive amplification for traC gene by the PCR-based plasmid typing with ND116-pRIV5-F1 and ND117-pRIV5-R1 primer pair.

Finally, as expected, 111 samples infected by Xf subsp. multiplex ST6 from Alicante (22 samples) and by Xf subsp. multiplex ST7 (3 samples) and ST81 (86 samples) from Mallorca and Menorca islands, as well as 40 samples infected by Xf subsp. pauca from Ibiza Island did not show amplification of the traC gene (Table 2).

2.3. Phylogenetic Analysis of traC Gene

Alignments of the sequences for all traC amplified products obtained from five Spanish strains of Xf subsp. fastidiosa ST1 from Mallorca Island (Table 1) or from eight plant samples naturally infected by this strain type, including three samples from almond trunk rings
indicated 100% homology among them and with strains Temecula1, TemeculaL, and M23 of Xf subsp. fastidiosa ST1, as well as Dixon strain of Xf subsp. multiplex ST6 (Figure S2). The traC sequences from the remaining Xf strains included in this study presented up to five different sequences (Figure S2). As expected, in silico analysis of the consensus traC gene sequence showed the best alignment score with the traC sequence of the pXFAS_5235 plasmid (GenBank accession NZ_CP047172, in which ND116-pRIV5-F1 and ND117-pRIV5-R1 primer pair start at position 3639 and 3157, respectively) and the pXF-RIV5 and pXFAS01 plasmids present on Xf Dixon and Bakersfield-1 strains belonging to the subsp. multiplex and fastidiosa, respectively.

BLAST search at NCBI, using as query the traC gene sequence of the pXFAS_5235 plasmid, matched with 58 drafts or completed whole-genomes of Xf with an identity between 95–99% and 99% query length coverage. As expected, the traC gene sequence showed 100% homology with a total of 14 genomes available from Spanish strains of Xf subsp. fastidiosa ST1 isolated from almond (7) and grape (7) from Mallorca Island. Interestingly, the traC gene was not exclusively found in Xf plasmids, but also on the chromosome of some Xf strains from Brazil: 3124, U24d, J1a12, 9a5c, and Hib4. For strain PD7211 of Xf subsp. paca ST73 intercepted in Netherlands, the traC gene was found in two different contigs: RRTZ0100001.1 and RRTZ0100002.1, both with 99% identity, but with 97.5% and 96.3% query length coverage, respectively.

Alignment of the traC gene of pXFAS-5235 against the RefSeq_genome and whole genome sequences databases revealed several polymorphisms between the traC gene sequence of strains belonging to the same subspecies fastidiosa, multiplex, and paca from different STs and geographic origins (Figure S2).

Maximum-likelihood phylogenetic tree (Figure 2) was built showing the different traC sequence types. The tree topology distinguished two main clusters (I and II) each with a bootstrap support of 99%, most of which were associated with different subspecies. Within cluster I, one of the main sub-clusters included all strains characterized as Xf subsp. fastidiosa ST1, with the exception of strain EB92.1 from Florida, USA, which was grouped in a second cluster with strains belonging to Xf subsp. fastidiosa ST2 (CFBP7970, CFBP8082, ATCC35879, and DSM_10026), which also originated from Florida. Moreover, this main cluster included Xf subsp. multiplex strains Dixon and RIV5. Interestingly, traC sequences from strains belonging to Xf subspecies paca ST53 appeared in the two main clusters and three different sub-clusters (Figure 2). Therefore, two strains of Xf subspecies paca ST53 (OLS0478 and OLS0479) from Costa Rica grouped with strain CFBP8478 of Xf subsp. sandy ST72 and the recombinant strain CO33 (93% bootstrap support), although their sequences presented some nucleotide polymorphisms (Figure S2). The next sub-cluster was constituted by the two strains of Xf subspecies paca ST53 (CFBP8495/ PD7202 and CFBP8429) isolated from Coffea arabica intercepted in Netherlands and France from plants from Costa Rica and grouped with strain CFBP8498/PD7211 of Xf subsp. paca ST73. The last sub-cluster from cluster I included all Xf subsp. multiplex ST87 from the outbreak of Tuscany in Italy (TOS4, 5, 14 and MA1, 10, 26, 151, 185) grouped in an independent cluster closely related to strain CFBP8498/PD7211 belonging to Xf subsp. paca ST73 from Costa Rica and strains CFBP8072 and CFBP8074 belonging to the Xf subspecies paca ST74 from Ecuador.

Cluster II was composed of three sub-clusters. The first one included several strains from diverse STs originated from Brazil and belonging to Xf subspecies paca ST11 (XRB, J1a12, B111), ST12 (CVC0251, CVC0256), ST13 (U24d, 9a5c), and ST16 (32, 3124). A second sub-cluster grouped all Xf subspecies paca ST53 from Italy and the coffee strain COF0407 from Costa Rica from the same ST53. The final sub-cluster close to this previous one included only Hib4 strain of Xf subspecies paca ST70 from Brazil.
Figure 2. Maximum-likelihood phylogeny showing the genetic relationships among \textit{traC} gene amplified from different \textit{Xylella fastidiosa} strains and naturally-infected plants from this study and from drafts or finished whole-genomes of \textit{Xf} present in the GenBank database. The numbers on the branches indicate the values of the bootstrap analyses.
3. Discussion

In this study, we have determined the presence of conjugative pXFAS_5235 plasmid in a collection of Xf strains and plant samples naturally infected by this bacterium obtained from the different outbreaks detected in Spain. In addition, throughout this work, we have assessed the incidence and distribution of the traC gene present in Xf strains belonging to different subspecies and STs from different regions of the world based on in-silico and PCR-based amplification. In this case, we developed a PCR-based typing approach using the primer pair (ND116-pRIV5-F1 and ND117-pRIV5-R1) that targets the traC gene within pXF-RIV5 plasmid from Xf subsp. multiplex strain RIV5 [23], whose sequence is nearly identical to pXFA01 from Xf subsp. fastidiosa strain M23 and pXFA5235 plasmid present in the Spanish strain IVIA5235 [22].

The sensitivity of the primer pair was evaluated in different plant DNA backgrounds calibrated at an initial concentration of $5 \times 10^5$ copies $\mu$L$^{-1}$. The fact that there are no differences between hosts in the sensitivity of the technique may be due to the use of BSA, which in other studies has been shown to compensate for polymerase inhibitors, and thus favor PCR efficiency [28]. In the same way, the gene could be amplified in naturally-infected samples from various hosts, where the lack of amplification was mainly related to a low concentration of bacterial cells (i.e., it was associated with high threshold values of Harper’s qPCR assay) [29].

Most of the Xf plant samples naturally infected by Xf subsp. fastidiosa ST1 amplified the traC gene, including DNA samples obtained from tree rings dated in 2004, which were previously shown as infected by Xf subsp. fastidiosa. The fact that in several tree ring samples known to be infected by Xf subsp. fastidiosa we could not achieve the traC could be due to the presence of PCR inhibitors, the type of sample processing (trunk vs. petiole) or a different DNA extraction protocol used for both plant tissues. An additional explanation, less plausible, may be that these rings were infected by a Xf subsp. fastidiosa strain not harboring the traC gene or the pXFA5235 plasmid. This strain, for some reason, was not established in the island or may have been overlooked.

Although the plasmid pXF64-Hb_ESVL from Xf subsp. multiplex ST6 showed a high sequence similarity to the conjugative plasmid pXF64-HB reported in Xf subsp. pauca ST70 [7,24,30], none of the samples characterized as infected by Xf subsp. multiplex ST6 from the Alicante focus (Valencian Community) amplified the traC gene. Since the role of traC is related to the family of conjugation proteins associated with the pilus structure [31], it would be interesting to study whether Xf subsp. multiplex ST6 conserves the complete set of potential conjugation genes to confirm whether this plasmid is capable of conjugative transfer, since this event plays a very important role in bacterial pathogenesis and evolution. However, pXF64-HB encodes several mating pair formation (MPF) proteins, including a MOBQ1 relaxase as well as one type IV coupling protein (T4CP) and one VirB4, which are key components of conjugative systems [24]. In addition, none of the Spanish strains or samples infected by Xf subsp. pauca ST80 from Ibiza Island showed amplification of the traC gene. Moreover, genome sequencing of the three isolates of Xf subsp pauca ST80 obtained from olive and almond trees in Ibiza Island have indicated that these strains do not harbor any plasmids (M.P. Velasco-Amo, L.F. Arias-Giraldo, and B.B. Landa, unpublished results).

Identical traC sequence was amplified for all strains or plant samples naturally infected by Xf subsp. fastidiosa ST1 obtained from the Balearic Islands. In addition, its sequence showed 100% similarity to traC sequence from several strains from California, USA, including strains of different Xf subspecies, such as pXFA01 in strains M23 and Bakersfield-1, pXFA01-RIV13 in strain RIV13 of Xf subsp. fastidiosa ST1, and pXF879-41 in strain ATCC-35879 of Xf subsp. fastidiosa ST2; pXF31k6 in Xf subsp. multiplex ST6 strain Dixon, pXF-RIV5 in Xf subsp. multiplex ST34 strain RIV5 [23], placing those strains in the same node of the traC ML phylogenetic tree (Figure 2). Similarly, Pierry et al. [24] found that ML phylogenetic trees of X. fastidiosa MOBP1 relaxases from plasmids were grouped as pXFA5235 with other plasmids from California, including pXF-RIV5, pXFA01, and pXF31k6. These results support the evidence of previous studies that pointed out to a single
introduction of *Xf* subsp. *fastidiosa* ST1 in the Balearic Islands with possible origin in the West Coast of the USA [16,18,19].

The *traC* gene was also amplified in strains of different *Xf* subsp. *pauca* from different STs (ST11, 12, 13, 16, 53, 70, 73, and 74), which were known to harbor a plasmid. However, there was a clear separation among the *traC* sequences found within the genome of *Xf* subsp. *pauca* strains, with those present in other subspecies, which agrees with the larger evolutionary distance of this subspecies as compared with subspecies *fastidiosa* and *multiplex*. Noticeably, up to three different *traC* sequences were found for strains belonging to *Xf* subsp. *pauca* ST53, which require further studies.

Interestingly, some *Xf* strains, whose complete genome is not available or its plasmid content is unknown, were amplified for *traC* gene, such as CFBP7970 and CFBP8082 from subsp. *fastidiosa*, Dixon from subsp. *multiplex*, CFBP8072 and CFBP8074 from subsp. *pauca*, and CFBP8478 from subsp *sandyi*. In these cases, an in-depth genomic analysis is required to determine whether this gene is conserved in the plasmid or whether it is inserted in the chromosome as with the strains 3124, U24d, J1a12, 9a5c, Hib4 from Brazil. Patterns of inter- and intrasubspecific homologous recombination between *Xf* subspecies showed that these Brazilian strains have a high number of recombination events [32,33] (M.P. Velasco-Amo, L.F. and B.B. Landa, unpublished results). Plasmid integration into the bacterial chromosome is quite common [26] and at least one fifth of the genes in the pangenome of *Xf* originated from distant branches of the evolutionary tree, which were presumably acquired by HGT [34]. The biologically relevant scenarios, in which plasmid transfer might have occurred between different strains of *Xf*, would be in the xylem vessels of the host plants or the insect vectors. In some cases, *Xf* strains belonging to different subspecies have been isolated from the same host plants growing in the same location, although it is unclear whether this is a common occurrence [35].

To conclude, the new developed PCR-based typing approach to determine the presence of p*XFAS_5235* plasmid and the ML phylogenetic analysis of *traC* revealed a close relatedness among Spanish and Californian strains carrying similar plasmids. Our results confirm previous studies, which suggested that a single introduction event of *Xf* subsp. *fastidiosa* ST1 occurred in the Balearic Islands.

### 4. Materials and Methods

#### 4.1. Bacterial and Plant Sample Sources and DNA Extraction Procedures

In this study, a total of 65 strains were used from the *Xf* collection at the Instituto de Agricultura Sostenible (IAS-CSIC), Córdoba, Spain belonging to subspecies *fastidiosa* (15 strains), *morus* (1 strain), *multiplex* (34 strains), *pauca* (11 strains), *sandyi* (3 strains), and a recombinant strain *pauca/sandyi* (Table 1). The strains isolated from the outbreaks in Alicante and Balearic islands in Spain were provided by different laboratories or acquired from the CFBP (Collection for Plant-associated Bacteria, INRAE, France). Strains were cultured in solid BCYE [36] or PD2 [37] solid media at 28 °C in the dark. After 7 to 12 days (depending on the strain), cells were collected for total DNA extraction using the DNeasy Plant Mini Kit (Qiagen). Total DNA were quantified in a ND-1000 spectrophotometer (Thermo Scientific™), and integrity was verified by 1% agarose gel electrophoresis.

A total of 203 plant samples naturally infected by *Xf* were obtained from different municipalities from Mallorca, Menorca, and Ibiza islands as well as from Alicante, Valencian Community, Spain, in the context of official monitoring surveys by the official diagnostic laboratories of Valencian Community and Balearic Islands in Spain (Table 2). Total plant DNA was extracted as described in the EPPO protocol [38] from leaf petioles or wood chip samples using the cetyltrimethylammonium bromide (CTAB)-based extraction or the EZNa HP Plant Mini kit (Omega-Biotek, Norcross, Georgia, USA), depending on the official laboratory providing the samples. DNA extracts were tested for the presence of *Xf* by Harper’s qPCR test with primers XF-F/XF-R and the dual-labeled probe XF-P [29], as described in [38]. Subspecies and ST assignment was performed using the conventional
MLST analysis [8,9] or a newly developed Nested-MLST analysis [28] at the Instituto de Agricultura Sostenible IAS-CSIC, Córdoba, Spain.

In addition, a total of 23 DNA plants samples obtained from tree rings from a previous study were used [20]. These samples included tree rings dated back to 1995, in which the Xf presence was detected using Harper’s qPCR test and the subspecies of Xf was typed using a multiplex qPCR [39], conventional MLST analysis [8] or Nested-MLST analysis [28] (Table 2).

4.2. PCR-Based Plasmid Typing

Initially, the 38 kb sequence of pXF-RIV5 plasmid (GenBank accession JX548317) from Xf subsp. multiplex strain RIV5 [23,40] was used as a template to design primers with Primer3 software [41]. Primers were originally designed to test for the presence of pXF-RIV5 plasmid among the different Xf infected samples from the outbreaks in Corsica and PACA region in France (Denancé, N., unpublished results). For primer design, default software parameters were selected, including optimal expected size (20 bp), Tm (60 °C), and GC content (50%), as well as a size product between 500 and 1 kb. The primers ND116-pRIV5-F1 (5′-ACACACTCGAAAGACC-3′) and ND117-pRIV5-R1 (5′-CACGCGGCTGATGAACATTA-3′) were selected, resulting in a yield of a 521 bp product. On the plasmid, ND116-pRIV5-F1 and ND117-pRIV5-R1 primer pair start at position 17,963 and 18,483, respectively, in traC gene involved in DNA replication and transcription (locus AGC23499; previously D698_p2018) [23]. The primers designed were checked by PCR for their ability to amplify the expected PCR product in Xf subsp. fastidiosa strain IVIA5235, once the availability of its genome revealed that this strain harbors the pX-FAS_5235 plasmid, whose sequence is nearly identical to the pXF-RIV5 plasmid [21,22]. Moreover, the Xf subsp. fastidiosa strain M23 that harbors the pXFAS01 plasmid was used for confirmation.

For typing of the traC gene, PCR assays were performed in a final volume of 20 µL, including 1 µM of primers, 0.5 µg/µL of BSA (Molecular Biology Grade, New England Biolabs), 5X of Taq DNA Polymerase buffer, and 1 U of My Taq™ DNA Polymerase (Bioline, London). The PCR was conducted in a T100 Thermal Cycler (BioRad, Hercules, CA, USA) using the following conditions: 94 °C for 3 min for initial denaturation; 34 cycles of 94 °C for 30 s, 60 °C for 30 s for annealing, and 72 °C for 45 s for strand elongation; at the end of all cycles, a step of 72 °C for 10 min for final elongation was performed. PCR products were evaluated by 1% agarose gel electrophoresis. DNA or boiled cells of Xf subsp. fastidious strain ST1 strain IVIA5235 harboring the pX-FAS_5235 plasmid was used as a positive control. In addition, sterile ultrapure demineralized water was used as a blank and synthetic DNA of Xf subsp. sandyi (GenomiPhiTM DNA Amplification Kit; Amersham Biosciences Piscataway, NJ, USA) as a negative control.

Few representative PCR products were selected for Sanger sequencing using the same primers at the STAB Vida Lda sequencing facilities (Caparica, Portugal). Sequences have been deposited on GenBank.

4.3. Limit of Detection of the PCR-Based Plasmid Typing

To establish the limit of detection of the developed PCR test for typing the presence of plasmid pXFA_5235 in plant DNA samples, two sets of DNA standard curves were obtained using 10-fold dilution series of Xf subsp. fastidious strain IVIA5235 DNA. For this purpose, Xf DNA from this strain was serially diluted in sterile ultrapure demineralized water as well as in a fixed background of host plant DNA (10 ng/µL) extracted from Prunus dulcis and Olea europaea wood chips, Vitis vinifera leaf petioles, Polygala myrtifolia leaf petioles or in a fixed background of insect DNA (10 ng/µL) from Neophilaenus campestris to obtain final concentrations starting from $5 \times 10^5$ up to 5 copies of Xf DNA/µL. Each standard curve always included plant or insect DNA alone as negative control. Prior to the preparation of standard curves, Xf DNA was quantified in triplicate using the Quant-iT™
PicoGreen™ dsDNA Assay Kit (Thermo Scientific™) and a Tecan Safire microplate reader (Tecan Group, Männedorf, Switzerland) to ensure accurate DNA concentrations.

Harper’s qPCR test [29] was performed on the same standard curves of Xf DNA in the different plants and insect DNA backgrounds to quantify the number of bacterial cells (copy number) and Ct values, at which the amplification of the pXFAS_5235 plasmid PCR signal was lost (i.e., limit of detection). The qPCR assays were performed on the thermal cycler LightCycler® 480, Roche Applied Science, using 96-well plates (fits LightCycler® 480 systems White, Landgraaf The Netherlands). The following thermal cycling program used was: 50 °C for 2 min, 95 °C for 10 min, then 45 cycles of two steps of 94 °C for 10 s and 62 °C for 40 s. The reaction mix was prepared in a final volume of 20 µL containing: iTaq™ Universal Probes Supermix (BioRad), 0.3 µM of each Xf forward and reverse primers (XF-F and XF-R, respectively), 0.1 µM of 6′FAM/BHQ-1 labeled probe (XF-P), 0.3 µg/µL of BSA (Molecular Biology Grade, New England Biolabs), and 2 µL of DNA sample. Three replicates of each concentration were tested simultaneously in the same run for each set of DNA standard curves. The data acquisitions and analyses were performed using LightCycler® 480 Software, v1.5. Linear regressions of the natural logarithm of known concentrations of the target DNA vs. the Ct values were performed for each DNA standard curve. Ct values up to 35 were considered as a positive result. Ct values between 35 and 38 were considered as inconclusive. Therefore, the sample was repeated and samples with Ct values higher than 38 were considered as not Xf infected.

4.4. Sequencing and Phylogenetic Analysis of traC

Sequence assembly for each sample was performed with Bionumerics v7.6 software package (Applied-Maths, Sint-Martens-Latem, Belgium). In addition, aligned sequences were submitted to BLAST searches for pairwise comparisons against the RefSeq_genome and whole genome sequences databases for Xf at GenBank.

Alignments of all traC sequences obtained in this study and those retrieved from GenBank were obtained using MAFFT [42], by selecting the “auto” mode for an appropriate alignment strategy. Subsequently, Spurious sequences or poorly aligned regions from the multiple sequence alignment were removed with trimAL v1.4.1 [43]. ModelTest-NG v0.1.6 [44] was executed with default parameters to select the appropriate substitution model for phylogenetic analyses. Based on the Bayesian information criterion (BIC) and the corrected Akaike information criterion (AIC), the TIM3ef + I + G substitution model was the best fit for our data out of the 80 DNA models tested. Finally, maximum-likelihood tree (ML) was computed using IQtree v2.2.0 [45] for DNA sequences with a 1000 bootstrap by the Ultrafast Bootstrap approximation approach (UFBoot) [46] and it was visualized with the R package ggtree v3.2.1 [47].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11121562/s1. Figure S1: Agarose gels showing traC gene amplification for selected X. fastidiosa strains from the collection at the Instituto de Agricultura Sostenible (IAS-CSIC) using ND116-pRIV5-F1/ND117-pRIV5-R1 primer pair (expected amplicon size of 521 bp). PAC: Positive amplification control contained boiled cells of IVIA5235 strain. NAC: Negative amplification control contained DNA from a Xf strain known not to harbor the traC gene. L: 10 kb GeneRuler™ DNA Ladder Mix (Thermo Scientific™); Figure S2: Alignments of all traC sequences obtained in this study and those retrieved from GenBank using MAFFT. Single nucleotide polymorphisms are indicated in relation to the consensus sequence. The subspecies of the Xf strain is indicated between brackets (F: fastidiosa, M: multiplex, P: pauca, M: morus, S: sandyi, S/M: sandyi/morus).

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