Development of A Nested-MultiLocus Sequence Typing Approach for A Highly Sensitive and Specific Identification of Xylella fastidiosa Subspecies Directly from Plant Samples

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Abstract: Identification of sequence types (ST) of Xylella fastidiosa based on direct MultiLocus Sequence Typing (MLST) of plant DNA samples is partly efficient. In order to improve the sensitivity of X. fastidiosa identification, we developed a direct nested-MLST assay on plant extracted DNA. This method was performed based on a largely used scheme targeting seven housekeeping gene (HKG) loci (cysG, gltT, holC, leuA, malF, nuoL, petC). Samples analyzed included 49 plant species and two insect species (Philaenus spumarius, Neophilaenus campestris) that were collected in 2017 (106 plant samples in France), in 2018 (162 plant samples in France, 40 plant samples and 26 insect samples in Spain), and in 2019 (30 plant samples in Spain). With the nested approach, a significant higher number of samples were amplified. The threshold was improved by 100 to 1000 times compared to conventional PCR. Using nested-MLST assay, plants that were not yet considered hosts tested positive and revealed novel alleles in France, whereas for Spanish samples it was possible to assign the subspecies or ST to samples considered as new hosts in Europe. Direct typing by nested-MLST from plant material has an increased sensitivity and may be useful for epidemiological purposes.

Keywords: Xylella fastidiosa; direct typing technique; MLST; nested PCR

1. Introduction

Xylella fastidiosa (Xf) is the causal agent of several devastating diseases of plants in the Americas and this pathogen was recently detected in Europe, where it causes a severe disease in olive trees in Italy and is present in several other regions. This species encompasses three well recognized subspecies, namely fastidiosa, multiplex, and pauca [1,2] but other subspecies are currently described [3]. The subspecies fastidiosa occurs in North and Central America and was recently detected in Spain (https://gd.eppo.int/taxon/XYLEFA/). It infects a wide range of trees, ornamentals, and other perennials and includes strains responsible for the well-known Pierce’s disease on grapevine [3,4]. The subspecies multiplex is present in North and South America and in Europe (https://gd.eppo.int/taxon/XYLEFA/) and
is associated with scorches and dieback of a wide range of trees and ornamentals [3]. The subspecies
pauca is mostly found in South and Central America on Citrus spp. and Coffea spp. [5]), but has
been recently detected also in olive trees in Spain (https://gd.eppo.int/taxon/XYLEFA/), Brazil [6],
Argentina [7], and Italy [8]. Its host range includes also ornamentals and other trees [3]. Altogether
more than 560 plant species are hosts of Xf [3]. This member of the Xanthomonadaceae family inhabits
the xylem of its host plants [9] and is naturally transmitted by insects from plants to plants.

A range of detection tests has been proposed for Xf [10]. Several immunological methods are
available [10]. However, such methods have high limits of detection (LoDs) that are close to 10^4 to
10^5 cells.mL^{-1} [10]. End point and also quantitative PCR (qPCR) are nowadays widely used, with a
better sensivity as the LoD is around 10^2 cells.mL^{-1} for several qPCR tests [11–14]. The Harper’s qPCR
test is often used in Europe for its high sensitivity. Several tests based on isothermal amplification
have also been reported [12,14–16]. The Harper’s test has also been successfully transferred to be
used in digital PCR [17]. Some of these tests were designed to detect only one subspecies. This is the
case of the nested PCR test proposed by [18] for detecting CVC (Citrus Variegated Chlorosis) strains
(subspecies pauca) in sharpshooters and citrus plants and also of the qPCR test targeting oleander leaf
scorch strains (that are included in the subspecies fastidiosa) [19]. Other tests were designed to detect
and discriminate two or more subspecies [16,20].

Precise identification of Xf at an infraspecific level is essential for epidemiological and surveillance
analyses, and to allow a proper description of the population structure and their dynamics. The widely
used MultiLocus Sequence Typing (MLST) scheme designed for Xf [21,22] is based on amplification by
conventional PCR and sequencing of seven HKG (housekeeping gene) fragments (loci), either from
strains or from plant samples [23]. For each locus, the different sequence variants are considered as
distinct alleles. The combination of allele numbers defines the sequence type (ST). The MLST-Xf data
are stored in a public database (https://pubmlst.org/xfastidiosa/) that can be used to automatically
identify and assign new allele variants, and provide tools to analyze the potential origin of the
strains. The association of the different subspecies with their host plants is useful to better understand
Xf epidemiology.

A reliable and enough informative typing method is particularly relevant in cases of new outbreaks
or for the description of new host. Due to the large number of host plants to be analyzed, various
types of inhibitors can interfere with reagents of PCR and low bacterial loads compromising PCR
efficiency and hence typing. Improving DNA extraction methods can, at least partly, solve the problem
of PCR inhibitors, and nested PCR appears a solution to allow the detection of low bacterial population
sizes. A nested-MLST was already successfully developed to detect and type Xf in vectors [24].
Primers were designed inside the gene fragments used in the conventional-MLST scheme and hence
some informative sites are lost. MLST with nested PCRs has also been developed in medical field to
enable the direct typing of samples infected by Leptospira or Trichomonas, for example [25,26].

The objective of this study was to develop a Xf detection assay based on the largely used MLST
scheme [22] that lowers the limit of detection (LoD) to enable at least the identification of Xf subspecies
and, if possible, provide larger sets of typing data directly from plant samples. We used genomic
sequences to improve each PCR efficiency and showed a drastic increase in the sensitivity as compared
to that of the conventional-MLST approach.

2. Materials and Methods

2.1. Strains and Media

A collection of target and non-target bacterial strains was used to test in vitro the specificity of
the newly designed primers and the nested PCR assays. This set was made of five X. fastidiosa strains
from different subspecies and 34 strains representing bacteria phylogenetically close to Xf, i.e., various
Xanthomonas, as well as strains of other plant pathogenic bacteria and endosymbionts potentially
inhabiting the same niches as \textit{Xf} (Table 1), available at the French Collection of Plant-Associated Bacteria (CIRM-CFBP; https://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria).

The \textit{Xf} strains were grown on modified PWG media (agar 12 g.L\(^{-1}\); soytone 4 g.L\(^{-1}\); bacto tryptone 1 g.L\(^{-1}\); MgSO\(_4\).7H\(_2\)O 0.4 g.L\(^{-1}\); K\(_2\)HPO\(_4\) 1.2 g.L\(^{-1}\); KH\(_2\)PO\(_4\) 1 g.L\(^{-1}\); hemin chloride (0.1% in NaOH 0.05 M) 10 mL.L\(^{-1}\); BSA (7.5%) 24 mL.L\(^{-1}\); L-glutamine 4 g.L\(^{-1}\)) at 28 °C for one week. \textit{Agrobacterium} and \textit{Rhizobium} were grown at 25 °C for one to two days on MG medium [27]; TSA was used (tryptone soybroth 30 g.L\(^{-1}\); agar 15 g.L\(^{-1}\)) for \textit{Clavibacter}, \textit{Ensifer}, \textit{Stenotrophomonas}, \textit{Xanthomonas} and \textit{Xylophilus}; and King’s medium B [28] for \textit{Dickeya}, \textit{Erwinia}, \textit{Pantoea} and \textit{Pseudomonas}. For PCRs, bacterial suspensions were prepared from fresh cultures in sterile distilled water, adjusted at \(\text{OD}_{600}\) nm = 0.1 and used as templates for amplification after boiling for 20 min, thermal shock on ice and centrifugation 10 000 g, 10 min.

2.2. DNA Extraction

Genomic DNA from \textit{Xf} strain CFBP 8070 was extracted with the Wizard genomic DNA Purification Kit (Promega, France) and used to prepare a 10-fold serial dilutions from 220 ng.mL\(^{-1}\) (corresponding to 8 \times 10\(^8\) copies.mL\(^{-1}\) of genomic DNA) to 22 fg.mL\(^{-1}\) (8 copies.mL\(^{-1}\)) to evaluate the LoD of the nested-MLST. Copies number were calculated using an estimated genome size of 2 903 976 bp, knowing that 1 pg = 9.78 \times 10\(^8\) bp [29]. A total of 268 plant samples were collected in Corsica, France, based on symptoms compatible with those caused by \textit{X. fastidiosa}; 106 samples were collected in June 2017 and 162 in September 2018. For each French sample, DNA was extracted as described in [10] using two methods in order to optimize the chances of detection. CTAB-based (Cetyl TrimethylAmmonium Bromide) extraction and robotic QuickpickTM SML kit from Bio-Nobile were used with the following modification: a sonication step (1 min, 42 KHz) was added after the samples (petioles, twigs) were finely cut, and was followed by a 15-min incubation period at room temperature. For initial laboratory diagnosis MLST results were compared with the Harper’s qPCR test [12] as in [10] with following modifications: primers XF-F and XF-R, and probe XF-P [12] were used at a final concentration of 0.6 \text{µM} and 0.2 \text{µM} respectively, non-acetylated BSA (Bovine serum Albumine) was used at final concentration of 1.5 \text{µg.µL}^{-1}, and 2 \text{µL} of DNA were used in 10 \text{µL} reaction volume. The target of this PCR is located in the gene coding for the 16S rRNA-processing RimM protein. Each DNA sample was tested in triplicates. To validate the nested PCR, DNA samples were provided by the National Reference Laboratory for Phytopathogenic Bacteria, Valencia, Spain, and from the Official Phytosanitary Laboratory of the Balearic Islands for determining \textit{Xf} subspecies. Those DNA samples correspond to DNA extractions made from symptomatic plants sampled during official monitoring surveys. A total of 70 \textit{Xf}-infected samples were analyzed from Balearic Islands and mainland Spain during 2018 (40 samples) and 2019 (30 samples), as well as 26 insect samples from both regions. DNA was extracted from petioles of symptomatic leaves as described in [10] using a CTAB-based extraction method for plant samples from Alicante and insect samples from Alicante and Balearic Islands. A Mericon DNeasy Food kit from Qiagen was used for plant samples from Balearic Islands. All DNA extraction methods have been validated; validation data is available in the EPPO (European and Mediterranean Plant Protection Organization) Database on Diagnostic Expertise [10].

2.3. Nested-MLST Primers and Reactions

The seven HKG sequences (\textit{cysG}, \textit{gltT}, \textit{holC}, \textit{leuA}, \textit{malF}, \textit{nuoL}, \textit{petC}) were extracted from 39 \textit{Xf} genome sequences (Table S1) [2] to design the nested primers. Alignments were performed with BioEdit sequence alignment editor. The primers designed by [22] were destined to be used as inner primers (PCR2) (Table 2) in our nested assay.
Table 1. List of target and non-target strains used to verify the specificity of nested-MLST primers.

| CFBP Code | Bacterial Species | Host Plant | Origin |
|-----------|-------------------|------------|--------|
| 6448      | Agrobacterium rubi | Rubus ursinus var. loganobaccus | USA (1942) |
| 2413      | Agrobacterium tumefaciens | Malus sp. | NA (1935) |
| 5523      | Agrobacterium vitis | Vitis vinifera | Australia (1977) |
| 2404      | Clavibacter insidiosus | Medicago sativa | USA (1955) |
| 4999      | Clavibacter michiganensis | Lycopersicon esculentum | Hungary (1957) |
| 3418      | Curtobacterium flaccumfaciens pv. flaccumfaciens | Phaseolus vulgaris | Hungary (1957) |
| 1200      | Dickeya dianthicola | Dianthus caryophyllus | United Kingdom (1956) |
| 3167      | Erwinia amplusora | Pyrus communis | United Kingdom (1959) |
| 3561      | Ensifer meliloti | Medicago sativa | VA, USA (1984) |
| 1392      | Erwinia amylovora | Prunus communis | Germany (1994) |
| 3205      | Pantoea agglomerans | Prunus persica | France (1974) |
| 3249      | Pantoea agglomerans | Prunus persica | Poland (2007) |
| 5241      | Xanthomonas campestris pv. campestris | Brassica oleracea var. gemmifera | United Kingdom (1997) |
| 3258      | Xanthomonas citri pv. aurantifolii | Citrus limon | Argentina (1988) |
| 3259      | Xanthomonas citri pv. citri | Citrus limon | New Zealand (1996) |
| 7660      | Xanthomonas citri pv. citicola | Vitis vinifera | India (1969) |
| 2625      | Xanthomonas gardneri | Medicago sativa | Reunion Island (1986) |
| 4295      | Xanthomonas hortorum pv. hederae | Hedera helix | USA (1944) |
| 2533      | Xanthomonas hortorum pv. pelargonii | Pelargonium peltatum | New Zealand (1974) |
| 1156      | Xanthomonas hyacinthi | Hyacinthus orientalis | Netherlands (1958) |
| 2532      | Xanthomonas oryzae pv. oryzae | Oriza sativa | India (1965) |
| 2054      | Xanthomonas translucens | Hordeum vulgare | USA (1933) |
| 2534      | Xanthomonas vasculum pv. holcicola | Sorghum vulgare | New Zealand (1969) |
| 7970      | Xylella fastidiosa subsp. fastidiosa | Vitis vinifera | USA (1987) |
| 8416      | Xylella fastidiosa subsp. multiplex | Polyspora myrtifolia | France (2015) |
| 8084      | Xylella fastidiosa subsp. morus | Morus alba | USA (na1) |
| 8070      | Xylella fastidiosa subsp. multiplex | Prunus spp. | USA (2004) |
| 8402      | Xylella fastidiosa subsp. panica | Olea europaea | Italy (2014) |
| 1192      | Xyleophilus ampelinius | Vitis vinifera | Greece (1966) |

1: not available.
Table 2. Primer sequences used in the *X. fastidiosa* nested-MLST scheme.

| Locus | PCR Round | 5'-Forward Primer-3' | 5'-Reverse Primer-3' | Position on *Xf* M12 Genome (CP000941.1) | Annealing Temperature (°C) | Size (pb) of Reaction Product |
|-------|-----------|---------------------|----------------------|------------------------------------------|---------------------------|-----------------------------|
| *cysG* | 1         | CCAAACATAGAGCAGACGCCG | GCGAGTGTTCATCGCCTCC  | 2111116–2111891                          | 64                        | 776                         |
|        | 2         | GCCGAAGCAGTGGGAAAG    | GCCATTTTCGATCAGTGCAAAG | 2111203–2111844                          | 56                        | 642                         |
| *gltT* | 1         | GGTGCCATCAATCCTGGTTT  | TCAGGATTCACCAATTCCTCC | 1731589–1732504                          | 60                        | 916                         |
|        | 2         | GCCGAAGCAGTGGGAAAG    | GCCATTTTCGATCAGTGCAAAG | 2111203–2111844                          | 56                        | 642                         |
| *holC* | 1         | CCGATGCTGGAGAAGAGTAGGA | GCTCGAGAAACTGGATAATTGG | 133166–133714                            | 62                        | 549                         |
|        | 2         | GGTCACATGTCGTGGGTTTT  | CACCGGCCGACTTCTATT    | 133269–133692                            | 59                        | 424                         |
| *leuA* | 1         | CGAAGGTCGACAAACAAATGGA | CGAATCGGCTTCGATAATGCT | 1271664–1272549                          | 58                        | 886                         |
|        | 2         | GGTGACGCGACAAATGGA    | ACTGGTCCCTGAACCTTCTCGT | 1271752–1272525                          | 60                        | 774                         |
| *malF* | 1         | AACGTCGTCACCCCAAGAAA  | ATGAGGCCGGCCTTTTGG    | 1680264–1681108                          | 56                        | 845                         |
|        | 2         | AGCAAGAAGCAGTGCCCATAT | CTGGTCTCGGCGGCTTGG    | 1680308–1681074                          | 60                        | 767                         |
| *nuoL* | 1         | TTGGATCGTTGGGTTGTG    | GACAAAACAGATTGGCGTC   | 325347–326191                            | 60                        | 845                         |
|        | 2         | GCGACTTACGGTACTGCGG   | ACCAGGATACCAAAGCAT1   | 325454–326050                            | 54                        | 597                         |
| *petC* | 1         | TCAATGACAGCCTCTCCTACA | GGCTGCAATCTGGTTAAGTA  | 2020498–2021079                          | 60                        | 582                         |
|        | 2         | ACGTCCTCCCAAATAAGGCT  | GGTATACGGTATCGGCTGC   | 2020505–2021055                          | 56                        | 551                         |

1: primers from [22].
We checked their characteristics with Primer3 V4.1.0 software (http://primer3.ut.ee/). Because of high Tm differences between forward and reverse primers for some primer pairs (gltT) (Table S2), or high hairpin Tm values (holC forward primer), some primers from [22] were redesigned nearly at the same positions to improve their efficiency. Moreover, as primer sequences were already near the locus sequence ends, we also had to relocate some of them to design nested primers inside the sequence alignments without loss of informative sites. Outer primers (PCR1) were designed with Primer3 V4.1.0 software (http://primer3.ut.ee/) in flanking regions targeted by the inner primers. Outer and inner primers were tested In silico using a primer search tool available in the galaxy toolbox of CIRM-CFBP (https://iris.angers.inra.fr/galaxypub-cfbp) on 194,438 bacterial Whole Genome Shotgun (WGS) sequences available in the NCBI database (as on March, 2019) including 58 Xylella and 1292 Xanthomonas (Table S3), and in vitro on target and non-target bacterial strains (Table 1).

PCRs were performed in 25 µL reaction buffer (Promega) with MgCl₂ at 1.5 mM final, 200 µM dNTP, 300 µM each of the forward and reverse primers, 0.6 U GoTaq G2 (Promega) and 2 µL of sample DNA. The first-round PCR program consisted of an initial denaturation step of 3 min at 95 °C followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at the relevant temperature according to each gene (determined by gradient PCR) and 60s elongation at 72 °C followed by a final extension step of 10 min at 72 °C (Table 2). The second round was performed with 30 cycles under same conditions and same concentrations but with a final volume of 50 µL for sequencing purposes and with 4 µL of first-round PCR product. The primer pairs of the second round of each nested PCR were used for sequencing (by Genoscreen, Lille, France for French samples and by Stabvida, Caparica, Portugal, for Spanish samples) the corresponding PCR products after 1.8% agarose gel visualization. To avoid contamination, one sample was opened at a time and stringent cleaning measures were applied after each experiment.

2.4. Statistical Analysis

The sensitivity of detection by conventional- and nested-MLST PCRs were compared in plant and vector samples for the seven HKGs that were analyzed by both approaches, by using a Chi square test using SAS (version 9.4, SAS Institute, Cary, NC, USA). Analysis was performed for the Spanish samples only, as HKG-PCRs were not systematically carried out on the French samples. Results were considered significantly different when \( p \leq 0.05 \).

2.5. Sequence Acquisition, Alignment and Analyses

Forward and reverse nucleotide sequences were assembled, and aligned using Geneious 9.1.8 software (French samples) or Bionumerics V7.6.3 software (Spanish samples) to obtain high quality sequences. ST or loci assignation was performed according to http://pubmlst.org/xfastidiosa/. To reduce the costs of sequencing for French samples, only PCR products obtained for samples showing the highest rate of successful HKG amplifications were sequenced. On the other hand, all positive holC amplifications were sequenced to obtain a larger view of alleles present in Corsica.

A flow chart summarizing the different steps of the nested-MLST method is presented in Figure 1.
3. Results

3.1. Nested-MLST Proved to be Specific

The specificity of the outer and inner primer pairs was tested In silico and in vitro. In silico, all primers pairs showed the best scores of alignment with Xf genomic sequences. Some non-target organisms showed sequences nearly identical at outer primer locations with only one mismatch and a similar expected fragment size, but sequences of inner primers were more different indicating that there will be no amplification. This was the case for various Xanthomonas strains that contained one mismatch at position 15 of the petC forward outer primer and an identical sequence for the outer reverse primer. X. taiwanensis holC sequence corresponding to inner primers contained also only one mismatch. The fragment size predicted was as expected for Xf. Other predictions with one mismatch located in primers did not end in fragment amplifications of the same expected size. Then, the specificity of the outer and inner primer pairs (Table 2) was validated in vitro on five target strains and 34 non-target strains (Table 1). Specificity of the nested-MLST assay could not been tested in vitro on X. taiwanensis as no strain was available. Amplifications were obtained for all Xf strains. No amplification was detected on the non-target strains except for strain CFBP 2532 (Xanthomonas oryzae pv oryzae) and CFBP 2533 (Xanthomonas hortorum pv. pelargonii) in the first round of the nested PCR for the petC outer primers, providing a product of the expected size. However, these products were not amplified in the second round of the nested PCR and no false positive signal was finally obtained.
3.2. Nested-MLST Limit of Detection is Comparable to That of qPCR

The sensitivity of each primer combination was evaluated on serial dilutions of a genomic DNA solution calibrated (Qubit fluorimeter, Invitrogen) at 220 ng.mL\(^{-1}\) (Figure 2). First round PCRs gave a signal more or less intense for concentrations up to 2.2 ng.mL\(^{-1}\) (\(0.8 \times 10^6\) copies.mL\(^{-1}\)) for all HKG except \(malf\) and \(cysG\) (220 pg.mL\(^{-1}\)). The second round of PCRs allowed a sufficiently strong signal for sequencing for concentrations up to 22 pg.mL\(^{-1}\) (\(0.8 \times 10^4\) copies.mL\(^{-1}\)) for \(gltT, holC, petC, leuA, cysG\), and up to 2.2 pg.mL\(^{-1}\) (\(0.8 \times 10^3\) copies.mL\(^{-1}\)) for \(nuoL\) and \(malf\).

![Detection threshold of conventional-MLST (a) and nested-MLST (b) for seven HKGs using genomic DNA dilution range (1:220 ng.mL\(^{-1}\); 2:22 ng.mL\(^{-1}\); 3:2.2 ng.mL\(^{-1}\); 4:220 pg.mL\(^{-1}\); 5:22 pg.mL\(^{-1}\); 6:2.2 pg.mL\(^{-1}\); 7:220 fg.mL\(^{-1}\); 8:22 fg.mL\(^{-1}\)).](image)

The same range of genomic DNA solutions was tested with the Harper’s qPCR test to compare sensitivity of these two tests (Table S4). The latest signal (LoD) for the Harper’s qPCR test (Cq = 37.64) was obtained with the concentration of 0.8.\(10^3\) copies.mL\(^{-1}\) and no amplification was obtained for lower concentrations.

Previously, we evaluated the LoD of the conventional PCRs for \(cysG\) and \(malf\) of the initial MLST scheme \([22]\) on a range of dilutions of CFBP 8070 genomic DNA with the Platinum Taq polymerase (Invitrogen) and tested the effect of adding BSA (final concentration at 0.3µg. µL\(^{-1}\)) on the efficiency of the conventional PCRs. No improvement was obtained as all signals remained around \(0.8 \times 10^6\) bacteria.mL\(^{-1}\) (Figure S1).

3.3. Analysis of Naturally Infected Samples

Using qPCR Harper’s test, 22 samples from 2017 and eight samples from 2018 collected in France were positive (Cq values < 35) with one or both DNA extraction methods; 70 samples from 2017 and 36 samples from 2018 were equivocal (35 ≤ Cq ≤ 40), 14 samples from 2017 and 118 from 2018 were negative (Cq > 40) (Table 3 and Table S5). Positive and equivocal samples were tested using the first round of PCR of the MLST assay: five samples from 2017 (one \(Spartium junceum\), three \(Polygala myrtifolia\), and one \(Genista corsica\)) gave a signal for at least one gene, but no complete typing was obtained for any sample. No sample from 2018 gave a signal. Most of Spanish samples used to evaluate nested-MLST scheme were positive using Harper’s qPCR (only two out of 40 plant samples were equivocal in 2018 and eight out of 26 vector samples).
Table 3. Number of samples, positive and equivocal in Harper’s qPCR. Percentage of successful amplifications obtained for each locus in conventional and nested PCR.

| Sample Type | Country | Year | Number of Samples | qPCR Harper Number of Samples | Percentage of Successful Amplifications Obtained for Each Locus in Conventional and Nested MLST-PCR | Average per Year |
|-------------|---------|------|-------------------|------------------------------|-----------------------------------------------------------------------------------------------|-----------------|
|             |         |      |                   | Cq < 35 | Cq ≥ 35 | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest | conv | nest |
| Plant       | France  | 2017 | 106               | 22      | 70      | 1.1  | 28.3 | 2.2  | 26.1 | 4.3  | 55.4 | 4.3  | 34.8 | 1.1  | 35.9 | 0    | 26.1 | 1.1  | 46.7 | 2    | 36.2 |      |      |      |      |      |      |
| Plant       | France  | 2018 | 162               | 8       | 36      | 0    | 11.4 | 0    | 9.1  | 0    | 27.3 | 0    | 27.3 | 0    | 15.9 | 0    | 27.3 | 0    | 25   | 0    | 20.5 |      |      |      |      |      |      |
| Plant       | Spain   | 2018 | 40                | 38      | 2       | 55   | 90   | 10   | 77.5 | 15   | 80   | 12.5 | 75   | 30   | 75   | 40   | 85   | 15   | 85   |      |      |      |      |      |      |
| Plant       | Spain   | 2019 | 30                | 30      | 0       | 30   | 90   | 13.3 | 90   | 16.7 | 93.3 | 16.7 | 90   | 20   | 90   | 66.7 | 90   | 20   | 90   | 26.2 | 90.5 |      |      |      |      |      |      |
| Insect      | Spain   | 2018 | 26                | 18      | 8       | 65.4 | 80.8 | 7.7  | 73.1 | 19.2 | 69.2 | 11.5 | 57.7 | 7.7  | 53.8 | 26.9 | 57.7 | 15.4 | 73.1 |      |      |      |      |      |      |

(*) Asterisk indicates a significant ($p < 0.05$) higher number of successful amplifications for nested-MLST as compared to conventional-MLST [22] according to a Chi-square test. The test was conducted only for the Spanish samples on the number of samples, even if frequencies are indicated in the table for MLST-PCR.
3.4. Nested-MLST Improved Successful HKG Typing by Increasing Sensitivity Level

Using nested-MLST for French samples, full allelic profiles were obtained for five samples from 2017 and one from 2018 corresponding to the lowest Cq in Harper’s qPCR test (Table 4 and Table S5). Among fully typed samples, four were X. fastidiosa subsp. multiplex ST7 (Genista corsica, Polygala myrtifolia, Spartium junceum), and two were X. fastidiosa subsp. multiplex ST6 (Polygala myrtifolia).

Our scheme was also evaluated on Spanish samples already proved infected by Xf. These samples from different outbreaks showed a wide range of Cq values ranging from 18.8 to 36.0 for plant samples and from 23.29 to 37.0 for insect samples (Table 3 and Table S5). Samples were first analyzed using the conventional-MLST assay [22]. Amplification efficiency was variable and ranged from 10% for gltT to 67% for nuoL with an average of 25% and 26% for the seven HKG in 2018 and 2019, respectively. The nested-MLST assay improved the amplification efficiency that increased to 75% for leuA and up to 93% for holC with an average of 81% and 91% in 2018 and 2019, respectively. In total, full allelic profiles were obtained in seven plant samples using the conventional-MLST assay, whereas a total of 55 samples were fully typed with the improved nested-MLST assay (Table 4). For the 70 plant DNA samples that were tested by both protocols, for all the seven HKGs, conventional-MLST showed a significant ($p < 0.0005$ for 2018 and $p < 0.0283$ for 2019) lower number of samples amplified as compared to nested-MLST. Among fully typed plant samples using the nested-MLST, we identified X. fastidiosa subsp. fastidiosa ST1 in Ficus carica and Juglans regia, X. fastidiosa subsp. multiplex ST6 in Helichrysum italicum, Olea europaea, Phagnalon saxatile, Polygala myrtifolia, Prunus armeniaca, Prunus domestica, Prunus dulcis, Rhamnus alaternus, and Rosmarinus officinalis, X. fastidiosa subsp. multiplex ST7 in Prunus dulcis, X. fastidiosa subsp. multiplex ST81 in Lavandula angustifolia and Prunus dulcis, and X. fastidiosa subsp. pauca ST80 in Cistus albidus, Prunus dulcis, and Rosmarinus officinalis.

Not all insect samples could be tested by both protocols due to restrictions in DNA amount. In samples tested only by the original MLST assay [22], the percentages of successful amplifications ranged from 8% (gltT and malF) to 65% (cysG). With the nested-MLST assay, successful amplifications ranged from 54% (malF) to 81% (cysG), with an average efficiency for the seven HKG of 22% to 67% for conventional and nested approach, respectively (Table 3 and Table S5). Nine insect samples were fully typed using a combination of both protocols (Table 4). X. fastidiosa subsp. fastidiosa ST1 was identified in insects from Mallorca (Balearic Islands), X. fastidiosa subsp. multiplex ST6 in insects from Alicante (mainland Spain) and X. fastidiosa subsp. multiplex ST81 in insects from Balearic Islands. For the nine insect samples that were tested by both protocols, conventional-MLST showed a significant ($p < 0.0247$) lower number of samples amplified as compared to nested-MLST for six of the seven HKGs (excluding cysG). These results indicate that for insect samples it is also better to use directly the improved nested-MLST assay.

No nonspecific amplicons were observed in any of the samples. Negative controls (water) were run in the first and the second PCR and were always negative. The negative control coming from the first reaction always tested negative in the second one, confirming the absence of contamination during the entire process. Positive control was a suspension of strain CFBP 8084 (ST29) from the subspecies morus or strain CO33 (ST72) as this STs were not previously found in Corsica, France or Spain, respectively.
Table 4. Allele numbers and STs obtained for fully typed samples in France and Spain for plant and insect samples. The numbers correspond to the names of the samples.

| Country | Sample Names          | cysG | gltT | holC | leuA | malF | nuoL | petC | Sequence Type (ST) |
|---------|----------------------|------|------|------|------|------|------|------|-------------------|
| France  | Spartium junceum 2   | 7    | 3    | 3    | 3    | 3    | 3    | 3    | ST7               |
| France  | Polygala myrtifolia 3, 4 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| France  | Genista corsica 1     | 7    | 3    | 3    | 3    | 3    | 3    | 3    | ST7               |
| France  | Polygala myrtifolia 5, 6 | 7    | 3    | 3    | 3    | 3    | 3    | 3    | ST7               |
| Spain   | Cistus albidus 2      | 31   | 15   | 10   | 7    | 17   | 16   | 6    | ST80              |
| Spain   | Ficus carica 1        | 1    | 1    | 1    | 1    | 1    | 1    | 1    | ST1               |
| Spain   | Helichrysum italicum 1 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Juglans regia 1       | 1    | 1    | 1    | 1    | 1    | 1    | 1    | ST1               |
| Spain   | Lavandula angustifolia 1 | 32   | 3    | 3    | 3    | 3    | 3    | 3    | ST81              |
| Spain   | Olea europaea 1       | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Phagnalon saxatile 1  | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Polygala myrtifolia 1 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Prunus armeniaca 1    | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Prunus domestica 1     | 32   | 3    | 3    | 3    | 3    | 3    | 3    | ST81              |
| Spain   | Prunus domestica 2     | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Prunus dulcis 4–8,10,11,15,18–26,30–47 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Prunus dulcis 9        | 31   | 15   | 10   | 7    | 17   | 16   | 6    | ST80              |
| Spain   | Prunus dulcis 1,2      | 32   | 3    | 3    | 3    | 3    | 3    | 3    | ST81              |
| Spain   | Prunus dulcis 3        | 7    | 3    | 3    | 3    | 3    | 3    | 3    | ST7               |
| Spain   | Rhamnus alaternus 1    | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Rosmarinus officinalis 4 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Rosmarinus officinalis 1,3 | 31   | 15   | 10   | 7    | 17   | 16   | 6    | ST80              |
| Spain   | Prunus domestica 3     | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Philaenus spumarius 6,7,8,10,11 | 1    | 1    | 1    | 1    | 1    | 1    | 1    | ST1               |
| Spain   | Philaenus spumarius 1  | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
| Spain   | Philaenus spumarius 22 | 32   | 3    | 3    | 3    | 3    | 3    | 3    | ST81              |
| Spain   | Neophilaenus campestris 1,2 | 3    | 3    | 3    | 3    | 3    | 3    | 3    | ST6               |
3.5. Nested-MLST Allowed Identification of New Alleles Among French Samples

Incomplete profiles were obtained for various French samples due to variable amplification efficiencies varying according to the HKG. From 9% (with gltT) to 55% (with holC) of French samples gave a signal applying the nested-MLST assay. Alleles that were not yet described in plant samples in France were detected in 2017. This was the case for holC_1 and holC_2 alleles known to occur in ST from ST1 to ST5 and ST75 that cluster in the subspecies fastidiosa (https://pubmlst.org/xfastidiosa/). These alleles were sequenced in samples of Asparagus acutifolius, Eleagnus, Cistus monspeliensis and C. creticus, Quercus ilex, Myrtus myrtifolia, Olea europaea, Platanus, Arbutus unedo (Table S5). Other holC alleles already described in STs clustering in the subspecies fastidiosa (holC_24) were also sequenced from Cistus monspeliensis and Pistacia lentiscus. HolC_10 alleles described in STs clustering in the subspecies pauca were sequenced from Cistus monspeliensis and C. salicifolius, Cypreus, Metroxideros excelsa, Myrtus communis, Pistacia lentiscus, Quercus ilex, Rubia peregrina, Smilax aspera samples. Similarly, holC_3 (known in ST6, ST7, ST23, ST34, ST35, ST79, ST81 and ST87 clustering in the subspecies multiplex) were obtained from samples of Acer monspeliensis, Arbutus unedo, Calicotome spinosa, Cistus monspeliensis, Genista corsica, Myrtus communis, Olea europaea, Phyllirea angustifolia, Polygala myrtifolia, Quercus ilex and Quercus pubescens, Spartium junceum. Among samples from 2018, only holC_1 allele was detected in Olea europaea, Quercus ilex, and Platanus sp. samples, and holC_3 allele in Cistus monspeliensis, Acer monspeliensis, Myrtus communis, and Polygala myrtifolia samples.

3.6. Recombinants or Mixed Infections Were Identified by Nested-MLST

Some French samples were further sequenced for several loci and these sequencing confirmed the presence of alleles occurring in the subspecies fastidiosa, multiplex and pauca (Table S5). All alleles were previously described but were detected in combinations that were not previously described, suggesting the presence of recombinants or of mix infections (Table S5). This is the case for Cistus monspeliensis 7 showing an unknown combination of cysG_2/petC_2/nuoL_2/gltT_2 (known in ST5) with malF_4 (known in ST2), both from subspecies fastidiosa; Helichrysum italicum 1 showing leuA_1 (known in subspecies fastidiosa) with petC_3/holC_3 known in subspecies multiplex; Myrtus communis 4 with leuA_3/holC_2 respectively known in subspecies multiplex and fastidiosa; Myrtus communis 8 and Platanus presenting form 1 alleles for five HKG mixed with malF_4 (all known in subspecies fastidiosa) and Q. ilex 10 presenting form 1 alleles for two HKG mixed with malF4); Olea europaea 2 with four multiplex alleles combined with nuoL_1 (subspecies fastidiosa); Olea europaea 5 with four pauca alleles combined with malF_15 (known in ST72 and ST76, subspecies fastidiosa). Two samples gave a double sequence for holC that were impossible to analyze (Table S5). Some sequences were ambiguous with superimposed peaks at some locations in otherwise good quality chromatograms revealing mixed infections. In those 12 samples, the number of potential combinations was too high to detect one probable allelic form, excepted for Prunus dulcis where the superimposed chromatograms corresponded to only two allelic forms (holC_3 or holC_6 which are found in subspecies multiplex). The holC_6 allelic form and the leuA_5 allele obtained for this sample are found in ST10, ST26, ST36, ST46, and ST63.

4. Discussion

A two-step nested procedure for MLST was developed to improve the typing of samples infected with low Xf population sizes that cannot be typed using the conventional protocol. In order not to affect the comparability of the results with the databases, the widely used MLST scheme developed for Xf that is supported by the pubMLST public website [22] was re-used.

The nested-MLST approach proved to be specific and efficient. No nonspecific amplifications were observed in any of the samples. Moreover, the sensitivities of the Harper’s qPCR detection test and the nested-MLST were similar with a LoD ranging from $10^3$ bacteria.mL$^{-1}$ to $10^4$ bacteria.mL$^{-1}$. These LoDs are similar to other nested-MLST approaches such as those developed for Burkholderia.
cepacia [30] but higher than for the one developed for Neisseria meningitidis (10 copies mL\(^{-1}\)) [31]. Consequently, in resource-limited settings where qPCR facilities are not available, the assay may be used as a useful diagnostic tool if applied with all necessary precautions to avoid cross-contamination between samples. The sequencing, which is costly, can be done as a consecutive but separate step to provide information on subspecies present in the sample. Higher bacterial loads (as indicated by lower Cq values) were observed in Spanish samples than in French samples, for which low amplification efficiency and partial profiles were observed. Full allelic profiles (ST6 and ST7 from multiplex subspecies) were obtained for Polygala myrtifolia, Spartium junceum and Genista corsica samples from France probably because they carried a higher bacterial load as shown by the low Cq obtained with the Harper’s qPCR test: five of the six typed samples had a Cq value between 23.4 and 26.5. The use of the nested-MLST assay to type plant Spanish samples allowed a higher number of successful complete typing (55 samples versus seven samples with the conventional approach). Spanish samples generally showed higher Xf titer (i.e., lower Cq values in Harper’s qPCR test) than the French samples but also concerned different plant species.

In our nested-MLST assay as well as in the original MLST assay, the amplification efficiencies were variable among genes, while all primers were designed using the same parameters from the software. For example, the holC gene for French samples tested with the nested-MLST assay was successfully amplified in 55% of samples collected in 2017 while the gltT and nuoL genes gave the lowest rates (around 26%). For samples collected in Spain tested with the original MLST assay, amplification rates among the seven HKGs ranged from 10 to 67%. Success rate variations were also observed in medical research using MLST between samples and between loci [25]. When conducted on strains, no differences about amplification rates are observed because of DNA excess. Robustness of a PCR reaction is determined by appropriate primers and it is not always obvious why some primer combinations do not amplify well, even if some parameters such as DNA folding can interfere in PCR efficiency [32]. In this study, even if primer annealing temperature was adjusted, design of primers was limited by their arbitrary localization.

Typing results of French samples were concordant with previously published results [23] but also revealed the presence of alleles not yet described in France. It should be noticed that no unknown sequence was obtained, refraining from evoking contaminations as the origin of these yet undescribed alleles in France. Thanks to the high rate of amplification of holC in nested PCR, it was also possible to obtain sequences for equivocal samples (Cq with the Harper’s qPCR test above 35) to confirm the presence of the bacterium in these samples. Surprisingly, these amplifications led to alleles that correspond to subspecies other than the multiplex subspecies. Thereby, alleles from subspecies pauca (holC_10) and fastidiosa (holC_1, holC_2, holC_24) were sequenced. holC_10 was already reported in Polygala myrtifolia in the south of France in 2015 [23]. holC_1 finding is in agreement with [24], who also reported holC_1 in insects in Corsica. Up to now, no holC_2 was reported in France but it is known in the USA. holC_24 was also reported in Polygala myrtifolia in Corsica in 2015 [23]. Further plant sampling efforts are needed to confirm the establishment of those strains in the environment or to document further the dynamics of alleles revealing sporadic infections.

For French samples only, several samples could not be typed since the chromatograms showed an overlap of two peaks precisely on the polymorphic sites (mainly with leuA and holC genes). This has already been reported by [23], it suggests the simultaneous presence of several strains in the same sample since only one copy of these genes are known in Xf [22]. Moreover, the report of previously unknown combination of alleles belonging to different subspecies can also results from the presence of co-infection or of recombinants. Recombination events are reported in Xf [23,33–35] and could have led to host shift [36]. In this study, eight samples presented unknown combinations of alleles from the same or different subspecies which could be explained by intrasubspecies or intersubspecies recombination events. As reported in [37], such events may exist and occur but not with the same frequency. Moreover, natural competence can be variable among Xf strains [38]. These events could also reflect a mechanism of adaptation [39]. Five samples among these eight samples were collected.
in 2017 and three in 2018, and were different between years. In 2018 the three cases were a similar combination of alleles and were found in three different plants. Future surveys will be necessary to know if some of these recombinants strains are indeed present in Corsica or are the consequence of mixed infections and if they have adapted and survived on different hosts.

The objective of this study was to improve the published MLST scheme supported by a public website (https://pubmlst.org/xfastidiosa) by designing nested primers to lower the limit of detection and help in \( Xf \) diagnosis and typing. Thus, this improved MLST assay enables a higher sensitivity and specific typing of \( Xf \) directly from plant and insects samples without the need of isolating the strain and at an affordable cost.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/8/1099/s1, Figure S1: detection threshold of conventional PCR for \( cysG \) and \( malF \) (Yuan et al., 2010) with and without BSA (final concentration at 0.3\( \mu \)g. \( \mu \)L\(^{-1} \) using genomic DNA dilution range (1: 220ng.mL\(^{-1} \); 2: 22ng.mL\(^{-1} \); 3: 2.2ng.mL\(^{-1} \); 4: 220pg.mL\(^{-1} \); 5: 22pg.mL\(^{-1} \); 6: 2.2pg.mL\(^{-1} \); 7: 220fg.mL\(^{-1} \); 8: 22fg.mL\(^{-1} \)). (+) positive control; (-) negative control, Table S1: List of \( X. fastidiosa \) genome sequences used in this study for primer and probe design (Denancé et al. 2019), Table S2: Primers properties, Table S3 Primer sequences alignment using a primer search tool available in the galaxy toolbox of CIRM-CFBP (https://iris.angers.inra.fr/galaxypub-cfbp) on 194438 bacterial Whole Genome Shotgun (WGS) sequences available in the NCBI database (as on March, 2019) including 58 \( Xylella \) and 1292 \( Xanthomonas \), Table S4: detection threshold for Harper’s qPCR test using genomic DNA dilution range (1: 220ng.mL\(^{-1} \); 2: 22ng.mL\(^{-1} \); 3: 2.2ng.mL\(^{-1} \); 4: 220pg.mL\(^{-1} \); 5: 22pg.mL\(^{-1} \); 6: 2.2pg.mL\(^{-1} \); 7: 220fg.mL\(^{-1} \); 8: 22fg.mL\(^{-1} \)), Table S5: results obtained with qPCR and nested-MLST. (+) means that a signal has been obtained in PCR but the PCR product has not been sequenced.

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Nomenclature

| Abbreviation | Description |
|--------------|-------------|
| BLAST | Basic Local Alignment Search Tool |
| Cq | quantification cycle |
| HKG | housekeeping gene |
| INRA | French National Institute for Agricultural Research |
| IRHS | Research Institute of Horticulture and Seeds |
| LoD | Limit of Detection |
| MLST | Multilocus Sequence Typing |
| NCBI | National Center for Biotechnology Information |
| ST | Sequence Type |
| Xf | \( Xylella fastidiosa \) |
| WGS | Whole Genome Shotgun |
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