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Genomics informed design of a suite of real-time PCR assays for the specific detection of each *Xylella fastidiosa* subspecies

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Xylella subspecies assays

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Abstract

Aims: Existing methods for the identification of the subspecies of *Xylella fastidiosa* are time consuming which can lead to delays in diagnosis and the associated plant health response to outbreaks and interceptions.

Methods and Results: Diagnostic markers were identified using a comparative genomics approach to allow fine differentiation of the very closely related subspecies. Five qPCR assays were designed to allow specific detection of *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *morus* and *X. fastidiosa* subsp. *sandyi*. All assays were validated according to the European and Mediterranean Plant Protection Organisation (EPPO) standard PM7/98(2).

Conclusions: All of the assays were shown to be specific to the target subspecies and all the assays could be used to detect femtogram quantities of *X. fastidiosa* DNA.

Significance and Impact of the Study: At present diagnosing the subspecies of *X. fastidiosa* requires multiple conventional PCR assays (although only available for 3 of the 5 subspecies) or multi locus sequence typing (MLST) which takes several days. By comparison the new assays provide a substantial reduction in the turnaround time for direct identification to the subspecies level in as little as 75 minutes.

Keywords: Diagnostics, plant pathogens, comparative genomics, MLST, subspecies identification

Introduction

Outbreaks and interceptions of plant pathogens occur with increasing frequency, often attributed to the increase in global trade of plant material and climate change altering natural climatic zones (Anderson *et al.*, 2004; Miller *et al.*, 2009). The impact of the spread of plant pathogens on a given ecosystem and economy can be great, as can the associated phytosanitary control measures (Alam and Rolfe, 2006; McCullough *et al.*, 2006). Accurate, rapid, detection and identification of pathogens is essential to prevent their introduction and dispersal into new areas and to allow exclusion and eradication measures for disease control (Miller *et al.*, 2009).

Xylella fastidiosa Wells *et al.*, (1987) is a Gram negative bacterium with fastidious growth requirements and is a regulated pathogen within the EU (Annex I/A/II, EPPO A2 list). It is limited to the xylem of plants and is vectored by xylem feeding insects where the bacteria grow in the foregut. The pathogen is endemic in the Americas (Jacques *et al.*, 2016) where it is associated with numerous plant diseases including Pierce's disease of grapevine and citrus variegated chlorosis (Janse and Obradovic, 2010). The taxonomy of *X. fastidiosa* is complex, with subspecies level descriptions. There are three formally described subspecies; *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, and *X. fastidiosa* subsp. *pauca* (Schaad *et al.*, 2004). A further two subspecies have been proposed but have an uncertain taxonomic standing; *X. fastidiosa* subsp. *sandyi* (Schuenzel *et al.*, 2005) and *X. fastidiosa* subsp. *morus* (Nunney *et al.*, 2014), and another has been referenced, *X. fastidiosa* subsp. *tashke* (Randall *et al.*, 2009). *X. fastidiosa* is unusual due to the lack of characteristic, diagnostic symptoms and high prevalence of asymptomatic infection in infected plants (EFSA, 2015). Typical symptoms such as leaf scorch, wilting, tip dieback and plant death are indistinguishable from those caused by numerous other plant pathogens and abiotic stressors (Wong *et al.*, 2003). Furthermore, dispersal over large areas is possible due to transmission by insect vectors and may be inadvertently aided by humans during propagation from infected (often asymptomatic) material (Almeida and Nunney, 2015; Denance *et al.*, 2017).

The first confirmed European finding of *X. fastidiosa* was in 2013 in Puglia, Italy, where *X. fastidiosa* subsp. *pauca* was found causing serious damage to olive groves (Saponari *et al.*, 2013). Subsequent findings in France (in 2015 on the mainland and Corsica) and Spain (in 2016 on the mainland and the Balearic islands (Mallorca, Menorca and Ibiza)) have led to all three formally

described subspecies being associated with outbreaks within Europe (European Commission, 2018). Furthermore, in 2016 an isolated finding of *X. fastidiosa* subsp. *fastidiosa* was reported in potted plants grown in a greenhouse in Germany (European Commission, 2018). Subsequent findings of *X. fastidiosa* subsp. *multiplex* in Tuscany, Italy (EPPO, 2019a) and Porto District, Portugal (EPPO, 2019b) and *X. fastidiosa* subsp. *fastidiosa* in the Hula Valley, Israel (EPPO, 2019c) are currently under containment/eradication. The bacterium has been detected in numerous host plants, with 595 species from 275 genera reported (EFSA, 2020). The detection of numerous subspecies within the European Union in recent outbreaks and interceptions highlights the need for easy, rapid detection to the subspecies to allow appropriate action to be taken. The subspecies is of relevance due to observed variations in plant host specificity, temperature ranges, vectors and geographic distribution between the subspecies (EFSA, 2015). For this reason, the subspecies of any given interception or outbreak is of interest from a plant health policy and bio-security perspective. Furthermore, there is a desire for this to be rapidly determined to allow the implementation of appropriate containment, control and/or eradication measures.

A range of diagnostic tools are available for *X. fastidiosa*, including culturing, serological methods (e.g. enzyme-linked immunosorbent assay (ELISA), direct tissue blot immunoassay (DTBIA), immunofluorescence microscopy (IF), and lateral flow devices (LFD)) and molecular methods (e.g. assays based on polymerase chain reaction (PCR), real-time PCR and loop-mediated isothermal amplification (LAMP)). Culturing from plant material is both very difficult, and time consuming due to the slow-growing nature of the bacterium (EPPO, 2018), and serological methods typically lack specificity and sensitivity (Vettraino *et al.*, 2010). Therefore, DNA extraction combined with molecular detection allows quicker and more accurate diagnosis (Hughes *et al.*, 2006). Due to the significance of a finding of any quarantine listed organism, a clear understanding of performance characteristics of the diagnostic assay is crucial. To this end, thorough validation prior to use is vital and over recent years there has been a move towards full validation of tests used within diagnostic laboratories. Plant pest and pathogen assays are often now validated in line with the EPPO standard PM9/78(2) (EPPO, 2014) which provides evidence of the tests suitability for use for a particular purpose and estimates of uncertainty associated with results (Clover *et al.*, 2010).

Whilst numerous different molecular methods have been developed over the years, many provide identification to the species, but not subspecies, level (e.g. Minsavage *et al.*, 1994). The most widely used molecular method is a ‘universal’ *X. fastidiosa* real-time PCR assay (Harper *et al.*, 2010, erratum 2013) which detects all subspecies, and remains useful as a first-line screen of suspect samples. Determination of the three formally described subspecies can be achieved by subspecies specific conventional PCR assays (Hernandez-Martinez *et al.*, 2006), although interpretation can be difficult and these may have poor performance characteristics (Li *et al.*, 2013). Furthermore, PCR assays are relatively time consuming to perform, pose a risk of laboratory contamination due to being an open-tube system and are less suited to high-throughput testing laboratories (Boonham *et al.*, 2008). A number of sub-species specific real-time PCR assays have been published (e.g. Ouyang *et al.*, (2013) for *X. fastidiosa* subsp. *pauca*, Burbank and Ortega (2018) for *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex*, and Dupas *et al.*, (2019) for *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca*, however there are not yet assays to detect all of the *X. fastidiosa* subspecies and the extent of validation data for existing published assays is variable hindering their deployment in diagnostic laboratories. Reliable identification to the subspecies level in all cases can be achieved by DNA sequencing of at least 7 genes (known as multi locus sequence typing/analysis, MLST/MLSA) (Scully *et al.*, 2005; Yuan *et al.*, 2010; Denance *et al.*, 2017), a time-consuming process which takes several days. Compared to other methods, real-time PCR confers numerous advantages such as reduction of laboratory contamination, higher sample throughput, reduced analysis time, increased sensitivity and increased specificity (Hughes *et al.*, 2006; Mumford *et al.*, 2006).

Differentiation of very closely related species, or taxonomic groups below the species level (e.g. pathovar, lineage or subspecies), is often challenging. Conventional design strategies employed for molecular diagnostics often do not allow the desired level of discrimination due to inadequate sequence differences in the commonly used, conserved genes. The rapid development of next-generation sequencing technologies over the past decade has led to a substantial increase in the availability of bacterial genome sequences (McAdam *et al.*, 2014). As such, an alternate approach being utilised is the comparison of genome sequence data to allow the identification of regions of DNA sequence which are unique, or substantially different between genomes, allowing the fine differentiation of very closely related isolates. This can be coupled with targeted assay design

strategies to allow the development of highly specific diagnostic assays. This approach has been used for plant pathogens, including *Xanthomonas arboricola* pv. *pruni* (Buhlman *et al.*, 2013; Garita-Cambronero *et al.*, 2017) and *Xanthomonas fragariae* (Gétaz *et al.*, 2017).

By utilising the latest genome comparison techniques and developing software to allow the identification of DNA markers with defined levels of sequence similarity, a suite of *Xylella fastidiosa* subspecies specific real-time PCR assays have been developed in this study. The new assays allow the rapid identification of all the described *X. fastidiosa* subspecies (*X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *morus* and *X. fastidiosa* subsp. *sandyi*), and have been validated according to the EPPO standard PM 7/98(2) (EPPO, 2014).

Materials and methods

Origin of samples

Xylella fastidiosa isolates (n=8) were sourced from the National Collection of Plant Pathogenic Bacteria (NCPBP, York, UK) and Belgian Co-ordinated Collections of Micro-organisms (BCCM-LMG, Ghent, Belgium). In addition, *X. taiwanensis* and 37 non-*Xylella* species (50 isolates) which are either common plant pathogens, endophytes or taxonomically closely related to *Xylella* were selected and sourced from the NCPBP or Fera collections. Isolates used in the study, their origin and original host are detailed in **Table 1**. Growth media and conditions used for the cultures are detailed in **Table S1**.

Healthy insects of the *X. fastidiosa* vector *Philaenus spumarius* (the meadow spittlebug) (2 specimens, both late instar nymphs) were collected from a lavender plant growing in East Yorkshire, UK, and screened for *X. fastidiosa* prior to use using the universal assay of Harper *et al.*, (2010) as described below.

Healthy plant material of 13 common *X. fastidiosa* hosts was sourced from UK plant nurseries and screened for *X. fastidiosa* prior to use using the universal assay of Harper *et al.*, (2010) as described below. Infected plant material was obtained from recent European outbreaks (naturally infected, from Corsica and Italy) and Costa Rica. Alternatively, healthy plants were artificially infected by inoculation with an aqueous suspension of cultured *X. fastidiosa* (strains NCPBP 4605 or NCPBP 4595 at 10^6 cfu's ml⁻¹) and grown under controlled environmental conditions in plant health quarantine licensed facilities (**Table 1**).

DNA extraction

DNA was extracted from a single colony of pure bacterial cultures using a QIAamp® DNA mini kit (QIAGEN, West Sussex, UK) following the manufactures protocol for spin columns. DNA was extracted from whole insect specimens using a DNeasy® blood and tissue kit (QIAGEN), following the manufactures protocol for animal tissues using spin columns. DNA was extracted from 0.3 g leaf material (healthy or infected) using a CTAB method based on that of Doyle and Doyle (1990) where the CTAB buffer was amended by the addition of 2 % polyvinylpyrrolidone (PVP-40) and 0.2 % sodium diethyldithiocarbamate and the removal of β-mercaptoethanol. DNA was quantified using a Qubit™ fluorometer with dsDNA high sensitivity kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) following the manufactures protocol.

Internal amplification controls

The plant cytochrome oxidase I (COX) gene real-time PCR assay of Weller *et al.*, (2000) as modified by Hughes *et al.*, (2006) was used to confirm the success of DNA extraction from plant samples. The assay was used as described in Hodgetts *et al.*, 2009.

PCR of approximately 1,200 bp of the 16S ribosomal RNA (rRNA) gene was performed on DNA extracted from bacterial cultures. PCR reactions (25 µl) comprised 1 µl DNA (concentration as extracted), 2x ReddyMix PCR Master Mix with 1.5 mM MgCl₂ (Thermo Fisher Scientific) and 400 nM each primer ufp1 (5' AGT TTG ATC CTG GCT CAG) and urp1 (5' GGT TAC CTT GTT ACG ACT T). PCR was performed in a GeneAmp™ 9700 thermal cycler (Applied Biosystems®, Foster City, California, USA) and cycling conditions were 95°C for 9 min followed by 35 cycles of 95°C for 1 min, 55°C for 2 min, 72°C for 1 min and a final extension of 72°C for 7 min. PCR products (5 µl) were separated by gel electrophoresis in 1 % agarose gel in 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), stained with ethidium bromide and visualised using a UV transilluminator.

PCR of approximately 700 bp of the cytochrome c oxidase subunit I (COI) gene was performed on DNA extracted from insects. PCR reactions (25 µl) comprised 12.5 µl 2x bio-x-act short PCR mix (Bioline, London, UK), 200 nM each primer LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G) and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer *et al.*, 1994), and 1.5 µl DNA (concentration as extracted). PCR was performed in a GeneAmp™ 9700 thermal cycler (Applied Biosystems®) and cycling conditions were 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 50 °C for 45 sec and 72 °C for 1 min, and a final extension of 72 °C for 10 min. PCR products were visualised as described above.

Genomic comparison

Genome assemblies were downloaded from GenBank® (2017), with 33 available for *X. fastidiosa* and a single outgroup genome for *X. taiwenensis*. (**Table S2**). Average nucleotide identity (ANI) between genomes was calculated using the ANIm method as described by Richter *et al.*, (2009). Briefly, all genome assemblies were compared against each other in a pairwise manner using NUCmer. Alignment lengths and similarity error counts were obtained for each alignment region between two assemblies and average nucleotide identity calculated between each pair of genomes. Visualisation of the ANI results with a heatmap and dendrogram were performed using the gplots package within the statistical software R (www.r-project.org).

For the discovery of sequences unique to each *Xylella* subspecies (or any required grouping of sequences/isolates, referred to as a cluster), a prototype software tool was developed and can be found at <http://www.github.com/rachelglover/FERA/>. Briefly, the software takes genome assemblies and a configuration file describing the expected clusters as input, divides the assemblies into a number of shorter DNA sequences (700 bp in length, referred to as ‘slices’) and determines the presence of each sequence in each assembly. This produced a number of sequences unique to each user-defined cluster to which assays could be designed.

Real-time PCR assay design

For each defined cluster (e.g. subspecies), putative markers were randomly selected and subjected to BLAST® searches at NCBI. Analogous sequences of the other *Xylella* subspecies were selected, downloaded and aligned (using MEGA v4.1, Tamura *et al.*, 2007) with those from the marker cluster. Each alignment was then manually assessed for suitability for design of a cluster specific real-time PCR assay. Markers with conserved sequence within the cluster, but with sequence variation between the other clusters were located, and the suitability of these markers for primer and probe design were assessed. Markers were assessed sequentially until one was located which showed probability of providing the desired assay specificity. Real-time PCR primers and probes were designed manually, using Primer Express v3.0.1 (Applied Biosystems®) to check oligo characteristics. Multiple assays were designed to multiple targets for each subspecies (**Table S3**) to exploit different regions of sequence variation. Primers and 5' FAM - 3' BHQ-1 labelled probes were purchased from Eurofins Genomics (Ebersberg, Germany), whilst 5' FAM - 3' MGB-NFQ probes were purchased from Applied Biosystems® (**Table 2**).

Real-time PCR

Preliminary evaluation of the specificity of all the designed assays was undertaken with a sub-set of bacterial species. Full evaluation and validation of the selected assay for each subspecies was performed.

Real-time PCR reactions were carried out in 96 well plates using the 7900 Real-Time PCR System (Applied Biosystems®, 2 machines). The C_T (cycle threshold) value for each reaction was determined using the Sequence Detection Software's default threshold setting. All samples were tested in duplicate (or triplicate for sensitivity evaluations), and all runs included no-template (negative) controls containing nuclease-free water in place of DNA and positive controls containing DNA extracted from pure cultures of *X. fastidiosa*.

Reactions (18 µl) comprised 2 µl DNA (concentration as extracted), 1x TaqMan Fast Universal PCR Master Mix no AmpErase® UNG (Applied Biosystems®), 6 µg ultrapure bovine serum albumen, 300 nM of each primer and 100 nM probe. The cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 10 sec and 62 °C for 40 sec. The universal assay of Harper *et al.*, (2010) was used as described above.

Assay validation

The assays were validated following the guidance provided in EPPO PM7/98(2) (EPPO, 2014). Analytical specificity and selectivity were evaluated by testing a range of *X. fastidiosa* isolates, other bacterial species, healthy insects, healthy and infected host plant species as described above. Analytical sensitivity of the assay was evaluated by testing three independent ten-fold dilution series of pure *X. fastidiosa* DNA diluted in water. Repeatability of the assay was evaluated by analysing 8 replicates of DNA extracts at three concentrations; neat DNA from pure bacterial cultures and DNA diluted to, and beyond, the assays limit of detection. For reproducibility testing, eight replicates were tested at three concentrations (as for repeatability) by two users on four different instruments (7500 Real-Time PCR System, 7900HT Real-Time PCR System (2 instruments), and ViiA™ 7 Real-Time PCR System, all Applied Biosystems®) on different days, with one platform tested by one user on two different days and a second user on a third day.

Results

Comparative genomic analysis

The ANIm genome comparison clustered the genome assemblies into three major groups (**Figure 1**), with further sub-divisions which present groupings which are primarily analogous to the formal taxonomic descriptions of *Xylella* subspecies. *X. fastidiosa* subsp. *pauca* is split into 3 clusters and *X. fastidiosa* subsp. *multiplex* into 2 clusters. Interestingly, *X. fastidiosa* subsp. *sandyi* and *X. fastidiosa* subsp. *morus* are combined into a single cluster closest to *X. fastidiosa* subsp. *fastidiosa*, although the isolates from each subspecies group with each other.

To identify possible molecular markers for specific assay design, the software was programmed to identify regions of sequence unique to each of the 5 *X. fastidiosa* subspecies (the user defined clusters) as well as the ANIm identified cluster of *X. fastidiosa* subsp. *sandyi* and *X. fastidiosa* subsp. *morus*. The number of unique markers identified by the software varied depending upon the inputted target; 125 for *X. fastidiosa* subsp. *fastidiosa*, 491 for *X. fastidiosa* subsp. *multiplex*, 6 for *X. fastidiosa* subsp. *pauca*, 45 for *X. fastidiosa* subsp. *morus*, 9 for *X. fastidiosa* subsp. *sandyi*, and 9 for *X. fastidiosa* subsp. *sandyi* with *X. fastidiosa* subsp. *morus*.

Real-time PCR assay design

Manual and *in silico* assessment of putative markers was undertaken for each cluster until those suitable for assay design were identified. Suitable markers showed sequence conserved within the isolates of the cluster but divergent from other clusters and other bacterial species. Due to the small differences observed between subspecies, most markers showed high levels of sequence identity between some, or all, of the subspecies, typically 89 - 95 %. Therefore, markers with multiple nucleotides conserved within the cluster and located close together were prioritised, to be exploited for assay design. For clusters where multiple promising markers were identified, assays were designed to each, and/or to different regions of the same marker, and/or with variations of primers. Analysis of the sequence differences within the primer and probe locations in non-target subspecies/species indicated the assays should provide specific detection. This resulted in 26 assay permutations; 5 for *X. fastidiosa* subsp. *fastidiosa*, 3 for *X. fastidiosa* subsp. *multiplex*, 1 for *X. fastidiosa* subsp. *pauca*, 9 for *X. fastidiosa* subsp. *morus*, 6 for *X. fastidiosa* subsp. *sandyi* and 2 for *X. fastidiosa* subsp. *sandyi* with *X. fastidiosa* subsp. *morus* (**Table S3**).

Preliminary real-time PCR assay evaluation

As a proof of principle test of the deployed marker identification and assay design strategy, an initial specificity assessment of all of the assays designed to the described subspecies was

undertaken. The initial specificity panel comprised representative members of the 5 *Xylella* subspecies and a small number of closely related plant pathogens (**Table 3**). Analysis of the assays demonstrated some were not specific to the desired subspecies, with 10 of the 24 assays showing cross-reaction with non-target DNA (**Table 3**). As an indicator of the extent of the observed cross-reaction, this was categorised as major ($C_T < 35$) or minor ($C_T > 35$), with 5 assays falling into each category. The majority of cross-reaction was seen within the other *X. fastidiosa* subspecies, with only one assay cross-reacting with a different bacterial genus (**Table 3**). Where assays were designed to multiple markers, the observed specificity varied considerably, including assays which differed by only a single primer (**Table 3** and **Table S3**).

Based on the preliminary assessment, at least one specific assay was developed for each subspecies, with 2 for *X. fastidiosa* subsp. *fastidiosa*, 2 for *X. fastidiosa* subsp. *multiplex*, 1 for *X. fastidiosa* subsp. *pauca*, 4 for *X. fastidiosa* subsp. *morus* and 5 for *X. fastidiosa* subsp. *sandyi*. Where multiple assays demonstrated the desired specificity, an assay resulting in a low C_T value with target DNA, with a typical sigmoidal amplification profile and a high fluorescent signal (ΔR_N value) was taken forward for full evaluation. The selected assays length, target gene region and percentage sequence identity to other subspecies over the length of the marker (approximately 700 bp, where sequence was available) were as follows; *X. fastidiosa* subsp. *fastidiosa*, 78 bp, *pilA*, $\leq 90\%$; *X. fastidiosa* subsp. *multiplex*, 85 bp, histone like protein, 90-91%; *X. fastidiosa* subsp. *pauca*, 103 bp, hypothetical protein, $\leq 94\%$; *X. fastidiosa* subsp. *morus*, 87 bp, putative hemagglutinin-related protein, $\leq 92\%$; *X. fastidiosa* subsp. *sandyi*, 50 bp, hypothetical protein, 91-95%.

Subspecies specific real-time PCR assay evaluation

The specificity of the selected assay for each subspecies was assessed against a large panel of non-target bacterial species including taxonomically closely related species, common plant pathogens, plant/soil endophytes, healthy insects, healthy and infected plant material (**Table 1**). Isolates of the target subspecies were tested and all were detected, giving C_T values in the range of 15.7 – 25.8 when testing DNA extracted from pure cultures. Specificity testing of each assay with the other *X. fastidiosa* subspecies showed that each assay only detected strains of the given subspecies, and all assays were demonstrated to be specific to the target subspecies. Further specificity assessments included testing 50 isolates (from 38 species) of non-target bacteria including *X. taiwanensis*, 1 insect species (2 specimens) and 13 healthy plant species. All non-target species produced negative results with all assays (**Table 1**). The ability of the assays to detect *in planta* infection

was demonstrated where material was available (**Table 1**), with C_T values ranging from 21.9 – 30.7 in naturally infected material, and 23.3 – 35.7 in artificially infected material. The success of DNA extraction and confirmation of the ability of the DNA extracts to support PCR was confirmed in all cases by positive results with the 16S rRNA conventional PCR assay (for bacteria cultures), COI conventional PCR assay (for insects), or the plant COX real-time PCR assay (for healthy and infected plant material) (data not shown).

The universal assay of Harper *et al.*, (2010) detected all isolates of *X. fastidiosa* (from all subspecies), producing C_T values in the range of 15.8 – 25.3 when testing DNA extracted from pure cultures. All non-target species (bacteria, insects and healthy plants) produced negative results. Detection *in planta* produced C_T values ranging from 21.8 – 32.6 in naturally infected material, and 23.4 – 36.5 in artificially infected material (**Table 1**).

The analytical sensitivity of each assay was determined using three independent 10-fold dilution series of DNA from pure culture of the target subspecies diluted in water, tested with three technical replicates. The assay limit of detection (LOD), defined as the lowest dilution where 100% of replicates were positive, ranged from 59.2 to 908 fg DNA per reaction, with C_T values in the range 32.2 to 35.1 (**Table 4** and **Table 5**). The LOD of the Harper *et al.*, (2010) universal assay (when tested with *X. fastidiosa* subsp. *fastidiosa*) was 124 fg DNA per reaction, with a C_T value of 31.4 (**Table 4** and **Table 5**). The LOD of the Harper *et al.*, (2010) universal assay was assessed separately with each of the *X. fastidiosa* subspecies and was found to range from 9.08 fg to 1.82 pg DNA per reaction, with C_T values at the LOD ranging from 31.5 to 36.7 (**Table 6** and **Table S4**).

A linear relationship between C_T value and DNA concentration with a high correlation coefficient was observed for all the assays tested (R^2 values from 0.9985 to 1.000), and the calculated assay amplification efficiencies ranged from 0.93 to 0.99 (**Table 4**).

For all assays, the diagnostic specificity and sensitivity were 100%, determined by testing healthy and infected plant samples. Furthermore under selectivity evaluation, there were no observable differences when testing in a range of plant species (**Table 1**).

Assay validation (EPPO)

The assays were validated according to the EPPO standard (**Table 7**). Repeatability and reproducibility was assessed by testing 8 replicates of DNA at different concentrations, by different users on different days and using different amplification equipment. DNA was tested at

high, low and minimal levels. At high and low levels of DNA all assays were 100% repeatable and reproducible with the exception of *X. fastidiosa* subsp. *morus* which was 100% repeatable; however not all replicates were positive at low levels of DNA when tested on one platform (Viia7). At minimal levels of DNA (at, to beyond, the assays limit of detection), data varied depending upon the assay, with reproducibility ranging from 8 to 63%. No effect of user or day was observed. At higher concentrations of DNA the different machines showed minimal variation, generally, the 7500 produced the lowest C_T values, and the Viia7 the highest (**Table 8**).

Identification of *X. fastidiosa* subspecies

Identification of *X. fastidiosa* subspecies requires preliminary screening, for example using a universal real-time PCR assay, followed by subspecies determination. MLST requires multiple conventional PCR assays (at least 7 genes), gel analysis, Sanger DNA sequencing and bioinformatics analysis, a process which takes a minimum of 3 to 4 days (**Figure 2**). Alternatively, testing a sample using the 5 subspecies specific real-time PCR assays (in parallel) would allow identification of the subspecies within 2 hours (**Figure 2**).

Discussion

Xylella fastidiosa is a bacterial disease with devastating impacts on plant based ecosystems, owing both to the disease itself and the associated plant health regulations. Due to the highly complex multifactorial pathosystem, with pathogen genetic variation, multiple hosts and a wide range of polyphagous vectors, few options exist for control. A number of established crop systems co-exist with the presence of the disease using cultural/management practices to maintain acceptable levels of productivity, for example in coffee and citrus production (Coletta-Filho *et al.*, 2013). Elsewhere, however, attention focuses primarily on control via exclusion and eradication, an approach that requires accurate diagnostic methods (Miller *et al.*, 2009). Diagnosis based on symptoms alone is not feasible due to the non-specific nature of the symptoms which can be confused with a myriad of other diseases, along with abiotic stressors such as temperature and water availability, both of which can also affect symptom expression. Furthermore, the low titre and uneven distribution of the pathogen within the plant frequently hamper effective detection (EPPO, 2016).

A large number of diagnostic tests have been developed for *X. fastidiosa* using a range of different detection technologies. Modern high-throughput testing laboratories frequently favour molecular based methods due to improved sensitivity, the ability to achieve specificity at a range of taxonomic levels and the associated benefits such as efficiency of testing (a range of real-time PCR tests for different targets can be designed to run under the same conditions) and the potential for automation (Mumford *et al.*, 2016). Real-time PCR is often the method of choice, due to its high sensitivity, speed of amplification and closed-tube analysis of results, which reduces the potential for contamination (Boonham *et al.*, 2008). Whilst screening for *X. fastidiosa* can currently be completed using real-time PCR assays, there are no assays available for the determination of all of the subspecies. Therefore, slower methods such Multi Locus Sequence Typing (MLST) are used where PCR amplification of multiple specific markers is followed by DNA sequencing and subsequent analysis of the sequence data. These methods are less suited to high-throughput testing, leading to delays in diagnosis which can be particularly frustrating to stakeholders (including policy makers, importers, growers etc.) involved in potential disease outbreaks of highly damaging and high profile pathogens (Rubio *et al.*, 2017).

By utilising the latest genome comparison techniques and developing software to allow the identification of molecular markers with defined levels of sequence similarity, a suite of *Xylella fastidiosa* subspecies specific real-time PCR assays have been developed. These assays provide

the required specificity to definitively identify *Xylella fastidiosa* subspecies in a format that is suited to delivering high-throughput and rapid turn-around results. Testing of these assays has demonstrated that subspecies specific assays have been successfully developed with this strategy. Assays have been developed for the three formally described species (*X. f.* subsp. *fastidiosa*, *X. f.* subsp. *multiplex* and *X. f.* subsp. *pauca*), as well as the proposed subspecies (*X. f.* subsp. *sandyi* and *X. f.* subsp. *morus*). Extensive specificity testing has demonstrated that the new assays are specific to the target subspecies. The sensitivity of all the assays was found to be comparable to the widely universal *X. fastidiosa* assay of Harper *et al.* (2010). Furthermore, the pathogen could be reliably detected in naturally and artificially infected plant material, demonstrating the suitability of the tests for screening plant material as well as confirming the identity of bacterial cultures. Whilst infected insect species were unavailable during the study, testing of healthy insects demonstrated a lack of cross-reactivity and indicates the likely suitability of the assays for detection within insects. The developed software, marker selection and assay design strategy could be further applied to other sequence data sets in the design of new diagnostics for other pathogen targets, using taxonomy based, or other user determined inputs, to identify markers with the desired specificity.

From the point of DNA extraction, the use of multi-locus sequence typing for subspecies determination takes a minimum of 3 working days (for PCR and DNA sequencing). In comparison, the use of the newly developed real-time PCR assays takes 75 minutes, substantially reducing the time required to determine the *X. fastidiosa* subspecies. With validation data in line with the EPPO standard and excellent performance characteristic, the newly developed assays complement the use of the widely used universal real-time PCR assay (Harper *et al.*, 2010) assay, enabling high-throughput screening for the presence of the pathogen, followed by rapid discrimination of *X. fastidiosa* subspecies in testing laboratories. Rapid and reliable detection and confirmation is essential, especially during the early stages of an outbreak of a quarantine pathogen or pest. Certainty in detection and identification facilitates rapid decision making as authorities seek to contain, prevent further spread and potentially eradicate damaging pathogens.

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Conflict of interest

No conflict of interest declared

Author contribution statement

JeH and NB conceived the research.

RG performed the genomic comparison. JH designed the assays. JeH, JC and JaH performed the experimental work.

JH wrote the first draft of the manuscript. NB critically reviewed the draft.

All authors read and approved the submitted version.

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List of figures, tables and supplementary material.

Figure 1. Average nucleotide identity (ANIm) genome comparison results. The heatmap demonstrates percentage identity between the two genomes with the percentage identity colours shown in the key. Black bars on the left indicate the *Xylella fastidiosa* subspecies; 'Xff' for *X. f.* subsp. *fastidiosa*, 'Xfmu' for *X. f.* subsp. *multiplex*, 'Xfp' for *X. f.* subsp. *pauca*, 'Xfs' for *X. f.* subsp. *sandyi*, and 'Xfmo' for *X. f.* subsp. *morus*. The outgroup (blue) is *Xylella taiwenensis*.

Figure 2. Comparison of the time required for determination of the *Xylella fastidiosa* sub-species using different identification methods. Green shading shows stages common to both methods, blue shading shows stages when using the newly developed real-time PCR assays and orange shading shows stages using conventional MLST analysis. DNA extraction time shows a range based on the extraction method selected. 'A' indicates analysis of real-time PCR results.

Table 1. Results of full specificity testing of the five *Xylella fastidiosa* subspecies specific real-time PCR assays and the universal *X. fastidiosa* assay (Harper *et al.*, 2010), with bacterial cultures, healthy insects, healthy and *X. fastidiosa* infected plant material. Data are average C_T values from two technical replicates, '-' indicates no amplification (C_T value recorded as 40.0). Grey shading indicates isolates of the target subspecies for a given assay. *NCPBP; National Collection of Plant Pathogenic Bacteria, LMG; (BCCM-LMG) Belgian Co-ordinated Collections of Micro-organisms.

Table 2. Primers and probes for the validated *Xylella fastidiosa* sub-species specific assay designed in the study

Table 3. Results of preliminary specificity testing of the complete set of newly developed subspecies real-time PCR assays. Grey shading indicates isolates of the target subspecies for a given assay. C_T values are the average of two technical replicates, '-' indicates no amplification (C_T value recorded as 40.0). *NCPBP; National Collection of Plant Pathogenic Bacteria, LMG; (BCCM-LMG) Belgian Co-ordinated Collections of Micro-organisms. † indicates the assay selected for complete validation.

Table 4. Results of sensitivity testing and assay efficiency for the 5 subspecies specific real-time PCR assays and the Harper universal assay (when tested with *X. fastidiosa* subsp. *fastidiosa*). Data is average values from 3 independent dilution series of DNA (in water), each tested with three technical replicates. The limit of detection (LOD) was defined as the lowest quantity of DNA where 100% of replicates were positive (n=9).

Table 5. Overall analysis of the subspecies specific real-time PCR assays and the Harper universal assay (when tested with *X. fastidiosa* subsp. *fastidiosa*) limit of detection. Three independent 10-fold dilution series were prepared in water from a single extract and tested on 3 different days by 1 user. Each dilution was tested in three technical replicates. The average C_T value from positive wells (any C_T value <40) and the number and percentage of positive replicates were calculated. Results are classified (shaded) as; no shading when 'positive' (all 3 replicates wells positive with C_T value <35; pale grey shading when 'tentative' (C_T value >35 or not all 3 replicate wells positive); dark grey shading when 'negative' (all 3 replicates with C_T value >40).

Table 6. Analysis of the Harper *et al.*, (2010) universal *X. fastidiosa* real-time PCR assay sensitivity with all of the *X. fastidiosa* subspecies. Dilution series (10-fold) were prepared in water and tested in three technical replicates. The limit of detection (LOD) was defined as the lowest quantity of DNA where 100% of replicates were classified as positive (n=3).

Table 7. Data on the validation of the subspecies specific real-time PCR assays according to the EPPO standard PM 7/98(2). Methods and results apply to all 5 developed assays.

Table 8. Full data from repeatability and reproducibility testing according to the EPPO standard PM 7/98(2). Eight technical replicates were tested for DNA concentrations obtained from pure cultures (neat), at (10^{-4} dilution), and beyond (10^{-5} dilution) the limit of detection for each assay. Four different machines were tested by two different users on three different days. Two machines of model 7900 were used indicated by the suffix 1 or 2. The left column shows the number of positive replicates. No shading demonstrates 100% of replicates produced positive results and shading indicates less than 100% of replicates produced positive results. The right column shows the average C_T value obtained (from positive replicates only), 'n/a' indicates all replicates were

negative (C_T value 40). *1 indicates the percentage of positive wells out of all tested replicates (n=48).

Supplementary material 1A. Details of the growth media and conditions for the bacterial species included in the study. See SM1B for the media composition. *NCPBP; National Collection of Plant Pathogenic Bacteria, LMG; (BCCM-LMG) Belgian Co-ordinated Collections of Micro-organisms. *1; unculturable species maintained in planta. *2; DNA extracted from freeze-dried culture.

Supplementary material 1B. Culture media compositions.

Supplementary material 2. Details of the genomes used in the comparative genomic analysis. Information regarding the isolate was collated from the NCBI accession information, or the associated reference where possible. * indicates plants tested positive in France after importation. ϕ indicates plant tested positive in Italy after importation. T indicates type strain. † indicates strain used both in the *in silico* and *in vitro* studies.

Supplementary material 3. Primer and probe sequences and assay performance characteristics for the complete set of assays designed in the study. The fully validated assay is indicated in column 'selected'. The specificity of each assay was assessed after preliminary specificity testing with eight *X. fastidiosa* isolates (representing 5 subspecies) and 4 non-target species (see Table 3 for details). Observed cross-reaction with non-target species or subspecies was classified as 'n/a' when not present, 'minor' when C_T value >35 and 'major' when C_T value <35.

Supplementary material 4. Analysis of the Harper *et al.*, (2010) universal *X. fastidiosa* real-time PCR assay sensitivity with all of the *X. fastidiosa* subspecies. A dilution series of DNA in water was tested, with data average values from three technical replicates. Results are classified (shaded) as; green when 'positive' (all 3 replicates wells positive with C_T value <35; yellow when 'tentative' (C_T value >35 or not all 3 replicate wells positive); red when 'negative' (all 3 replicates with C_T value >40). The limit of detection was defined as the lowest quantity of DNA where 100% of replicates were classified as positive.

Table 1. Results of full specificity testing of the five *Xylella fastidiosa* subspecies specific real-time PCR assays and the universal *X. fastidiosa* assay (Harper *et al.*, 2010), with bacterial cultures, healthy insects and *X. fastidiosa* infected plant material. Data are average C_T values from two technical replicates, '-' indicates no amplification (C_T value recorded as 40.0). Grey shading indicates isolates of the target subspecies for a given assay. *NCPBPB; National Collection of Plant Pathogenic Bacteria, LMG; (BCCM-LMG) Belgian Co-ordinated Collections of Micro-organisms.

Isolate reference*	Species	Country of origin	Host	<i>X. f. subsp. fastidiosa</i>	<i>X. f. subsp. multiplex</i>	<i>X. f. subsp. pauca</i>	<i>X. f. subsp. morus</i>	<i>X. f. subsp. sandyi</i>	Harper
<i>Xylella fastidiosa</i>									
LMG 17159	<i>X. f. subsp. fastidiosa</i>	USA	Grapevine	16.7	-	-	-	-	17.2
LMG 15099	<i>X. f. subsp. fastidiosa</i>	USA	Almond	16.1	-	-	-	-	15.8
NCPBPB 4605	<i>X. f. subsp. fastidiosa</i>	USA	<i>Vitis vinifera</i>	16.0	-	-	-	-	16.6
LMG 9063	<i>X. f. subsp. multiplex</i>	USA	<i>Prunus salicina</i>	-	25.8	-	-	-	25.3
NCPBPB 4604	<i>X. f. subsp. multiplex</i>	USA	<i>Vaccinium</i> sp. (blueberry)	-	18.1	-	-	-	17.4
NCPBPB 4595	<i>X. f. subsp. pauca</i>	Italy	<i>Olea europea</i>	-	-	16.9	-	-	18.0
NCPBPB 4589	<i>X. f. subsp. morus</i>	USA	<i>Morus</i> sp.	-	-	-	16.7	-	16.3
NCPBPB 4606	<i>X. f. subsp. sandyi</i>	USA	<i>Nerium oleander</i>	-	-	-	-	15.7	16.1
Other bacterial species									
NCPBPB 2889	<i>Burkholderia</i> sp.	France	<i>Lycopersicon esculentum</i>	-	-	-	-	-	-

n/a	' <i>Ca. Liberibacter africanus</i> '	South Africa	<i>Citrus</i> sp.	-	-	-	-	-	-
n/a	' <i>Ca. Liberibacter solanacearum</i> '	Unknown	<i>Daucus carota</i> subsp. <i>sativus</i>	-	-	-	-	-	-
n/a	' <i>Ca. Phytoplasma asteris</i> '	Czech Republic	<i>Catharanthus roseus</i>	-	-	-	-	-	-
n/a	' <i>Ca. Phytoplasma ulmi</i> '	Italy	<i>Ulmus</i> cv. Morfeo	-	-	-	-	-	-
NCPPB 3896	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	UK (Scotland)	<i>Solanum tuberosum</i> cv. Kievsky	-	-	-	-	-	-
			tvitamok	-	-	-	-	-	-
NCPPB 4048	<i>Cupriavidus necator</i>	USA	Soil	-	-	-	-	-	-
NCPPB 4168	<i>Enterobacter</i> sp.	Unknown	Unknown	-	-	-	-	-	-
NCPPB 4170	<i>Ochrobacterium anthropi</i>	Unknown	Unknown	-	-	-	-	-	-
NCPPB 4162	<i>Paenibacillus polymyxa</i>	France	<i>Solanum tuberosum</i>	-	-	-	-	-	-
NCPPB 1931	<i>Pantoea agglomerans</i>	New Zealand	<i>Cosmos sulfureus</i>	-	-	-	-	-	-
NCPPB 4163	<i>Pseudomonas marginalis</i> pv. <i>marginalis</i>	USA	<i>Solanum tuberosum</i>	-	-	-	-	-	-

NCPPB 3686	<i>Pseudomonas syringae</i> pv. <i>persicae</i>	New Zealand	<i>Prunus salicina</i>	-	-	-	-	-	-
NCPPB 3687	<i>Pseudomonas syringae</i> pv. <i>persicae</i>	New Zealand	<i>Prunus salicina</i>	-	-	-	-	-	-
NCPPB 3899	<i>Ralstonia pickettii</i>	Unknown	Unknown	-	-	-	-	-	-
NCPPB 787	<i>Ralstonia solanacearum</i>	Costa Rica	<i>Musa</i> sp.	-	-	-	-	-	-
NCPPB 3205	<i>Ralstonia solanacearum</i>	Guyana	<i>Musa</i> sp. (plantain)	-	-	-	-	-	-
NCPPB 3792	<i>Ralstonia syzygii</i>	Central Java	<i>Syzigium aromaticum</i>	-	-	-	-	-	-
NCPPB 3726	<i>Ralstonia syzygii</i> subsp. <i>celebesensis</i>	South Sulawesi	<i>Musa</i> hybrid banana	-	-	-	-	-	-
NCPPB 2404	<i>Rhizobium radiobacter</i>	Australia	<i>Prunus persica</i>	-	-	-	-	-	-
NCPPB 2562	<i>Rhizobium vitis</i>	Greece	<i>Vitis vinifera</i>	-	-	-	-	-	-
NCPPB 3269	<i>Rhizobium vitis</i>	Afghanistan	<i>Vitis vinifera</i>	-	-	-	-	-	-
NCPPB 3554	<i>Rhizobium vitis</i>	Australia	<i>Vitis vinifera</i>	-	-	-	-	-	-
NCPPB 2565	<i>Spiroplasma citri</i>	Israel	<i>Citrus sinensis</i>	-	-	-	-	-	-
NCPPB 887	<i>Xanthomonas albilineans</i>	Mauritius	<i>Saccharum officinarum</i>	-	-	-	-	-	-
NCPPB 1630	<i>Xanthomonas arboricola</i> pv. <i>celebensis</i>	New Zealand	<i>Musa</i> sp.	-	-	-	-	-	-

NCPPB 4183	<i>Xanthomonas arboricola</i> pv. <i>fragariae</i>	Italy	<i>Fragaria x</i> <i>ananassa</i>	-	-	-	-	-
NCPPB 411	<i>Xanthomonas arboricola</i> pv. <i>juglandis</i>	New Zealand	<i>Juglans regia</i>	-	-	-	-	-
NCPPB 1833	<i>Xanthomonas axonopodis</i> pv. <i>dieffenbachiae</i>	Brazil	<i>Anthurium</i> sp.	-	-	-	-	-
NCPPB 3380	<i>Xanthomonas axonopodis</i> pv. <i>dieffenbachiae</i>	Jamaica	<i>Anthurium</i> sp.	-	-	-	-	-
NCPPB 186	<i>Xanthomonas axonopodis</i> pv. <i>vasculorum</i>	Mauritius	<i>Thysanolaena</i> <i>maxima</i>	-	-	-	-	-
NCPPB 899	<i>Xanthomonas axonopodis</i> pv. <i>vasculorum</i>	Reunion	<i>Saccharum</i> <i>officinarum</i>	-	-	-	-	-
NCPPB 45	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	UK	<i>Brassica napus</i>	-	-	-	-	-
NCPPB 529	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	UK	<i>Brassica</i> <i>oleracea</i> var. <i>capitata</i>	-	-	-	-	-
NCPPB 4389	<i>Xanthomonas campestris</i> pv. <i>musacearum</i>	Rwanda	<i>Musa</i>	-	-	-	-	-
NCPPB 4387	<i>Xanthomonas campestris</i> pv. <i>musacearum</i>	D.R.Congo	<i>Musa</i>	-	-	-	-	-

NCPPB 409	<i>Xanthomonas citri</i>	New Zealand	-	-	-	-	-	-	-
NCPPB 3799	<i>Xanthomonas citri</i> subsp. <i>citri</i>	UK	-	-	-	-	-	-	-
NCPPB 3233	<i>Xanthomonas fuscans</i> subsp. <i>aurantifolia</i>	Brazil	-	-	-	-	-	-	-
NCPPB 2985	<i>Xanthomonas hortorum</i> pv. <i>pelargonii</i>	New Zealand	-	-	-	-	-	-	-
NCPPB 4031	<i>Xanthomonas hortorum</i> pv. <i>pelargonii</i>	UK	-	-	-	-	-	-	-
NCPPB 3002	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	India	-	-	-	-	-	-	-
NCPPB 4321	<i>Xanthomonas perforans</i>	USA	-	-	-	-	-	-	-
NCPPB 989	<i>Xanthomonas vasicola</i> pv. <i>holcicola</i>	USA	-	-	-	-	-	-	-
NCPPB 1060	<i>Xanthomonas vasicola</i> pv. <i>holcicola</i>	Ethiopia	-	-	-	-	-	-	-
NCPPB 206	<i>Xanthomonas vasicola</i> pv. <i>vasculorum</i>	South Africa	-	-	-	-	-	-	-
NCPPB 702	<i>Xanthomonas vasicola</i> pv.	Zimbabwe	-	-	-	-	-	-	-

	vascularum						officinarium
NCPBPB 422	Xanthomonas vesicatoria	New Zealand	-	-	-	-	Lycopersicon escutentum
NCPBPB 701	Xanthomonas vesicatoria	Zimbabwe	-	-	-	-	Lycopersicon escutentum
NCPBPB 4612	Xylolla taiwanensis	Taiwan	-	-	-	-	Pyrus pyrifolia
Healthy insects							
n/a	Philaenus spumarius	UK	-	-	-	-	Lavender
n/a	Philaenus spumarius	UK	-	-	-	-	Lavender
Healthy plants							
n/a	n/a	UK	-	-	-	-	Catharanthus roseus
n/a	n/a	UK	-	-	-	-	Citrus sp. (lemon)
n/a	n/a	UK	-	-	-	-	Cistus x pulverulentus
n/a	n/a	The Netherlands	-	-	-	-	'Sunset'
n/a	n/a	The Netherlands	-	-	-	-	Coffea arabica
n/a	n/a	The Netherlands	-	-	-	-	Lavandula angustifolia

n/a	n/a	Spain	<i>Nerium oleander</i>	-	-	-	-	-	-
n/a	n/a	Italy	<i>Polygala myrtifolia</i>	-	-	-	-	-	-
n/a	n/a	UK	<i>Olea europaea subsp. europaea</i>	-	-	-	-	-	-
n/a	n/a	Italy	<i>Quercus ilex</i>	-	-	-	-	-	-
n/a	n/a	Italy	<i>Rosmarinus officinalis</i>	-	-	-	-	-	-
n/a	n/a	UK	<i>Ulmus</i> sp.	-	-	-	-	-	-
n/a	n/a	UK	<i>Vinca minor</i>	-	-	-	-	-	-
n/a	n/a	Italy	<i>Vitis coignetiae</i>	-	-	-	-	-	-
Infected plants									
n/a	<i>X. f.</i> subsp <i>fastidiosa</i>	Artificially infected	<i>Laurus nobilis</i>	35.7	-	-	-	-	36.5
n/a	<i>X. f.</i> subsp <i>fastidiosa</i>	Artificially infected	<i>Catharanthus roseus</i>	23.3	-	-	-	-	23.4
n/a	<i>X. f.</i> subsp <i>fastidiosa</i>	Artificially infected	<i>Myrtus communis</i>	31.2	-	-	-	-	31.9

n/a	<i>X. f.</i> subsp. <i>multiplex</i>	Corsica	<i>Genista</i> sp.	-	26.9	-	-	27.2
n/a	<i>X. f.</i> subsp. <i>multiplex</i>	Corsica	<i>Helichrysum</i> sp.	-	22.1	-	-	23.2
n/a	<i>X. f.</i> subsp. <i>multiplex</i>	Corsica	<i>Calicotome</i> sp.	-	21.9	-	-	21.8
n/a	<i>X. f.</i> subsp. <i>pauca</i>	Italy	Polygala	-	-	30.7	-	32.6
n/a	<i>X. f.</i> subsp. <i>pauca</i>	Italy	Olive	-	-	24.7	-	25.9
n/a	<i>X. f.</i> subsp. <i>pauca</i>	Artificially infected	<i>Catharanthus roseus</i>	-	-	29.2	-	30.7
n/a	<i>X. f.</i> subsp. <i>pauca</i>	Artificially infected	<i>Rosmarinus officinalis</i>	-	-	31.6	-	30.9
n/a	<i>X. f.</i> subsp. <i>sandyi</i>	Costa Rica	<i>Coffea arabica</i>	-	-	-	26.9	28.3

Table 2. Primers and probes for the validated *Xylella fastidiosa* sub-species specific assay designed in the study

Target	Element	Sequence (5'-3')	Reporter-quencher
<i>X. f.</i> subsp. <i>fastidiosa</i>	Xff_3-Fw-a_122365	TCGTTGTGGGATTACCGTTAAA	
	Xff_3-Rv_122365	ACCTGAGAAATTGCCCTTAATCG	
	Xff_3-Pr_122365	TCGAAAAACACCGGACTTGCCAAACA	FAM-BHQ1
<i>X. f.</i> subsp. <i>multiplex</i>	Xfmu_3-Fw_15747	CAATCGCTTTTGAGGTCAATCC	
	Xfmu_3-Rv_15747	GCGATTGTTTCTTCTCTACACCAAG	
	Xfmu_3-Pr_15747	TCTGCAAAACGCTTTAAAAAACTGCTCGCC	FAM-BHQ1
<i>X. f.</i> subsp. <i>pauca</i>	Xfp_1-Fw_28775	GCATCCTCACCCACCGAAGG	
	Xfp_1-Rw_28775	TCCACATCCAGCAAGGTGAC	
	Xfp_1-Pr_28775	CCTTGGACGCGGATACCCGCA	FAM-BHQ1
	Xfmo_7_Fw_67218	CCACCTCGCTTTTAGTTACGTGATT	
<i>X. f.</i> subsp. <i>sandyi</i>	Xfmo_7_Rva_67218	GGAGTTTATTGGCTGAACTGAGTG	
	Xfmo_7_Pr_67218	AAGCGTGATACTACTCC	FAM-MGB-NFQ
	Xfs_4_Fwb_112076	CCCCGCTGTGGCAGAA	
<i>X. f.</i> subsp. <i>sandyi</i>	Xfs_4_Rv_112076	GGTCCGAGCCATACGGC	
	Xfs_4_Pr_112076	CAGCGCCTTCAATC	FAM-MGB-NFQ

Table 3. Results of preliminary specificity testing of the complete set of newly developed subspecies real-time PCR assays. Grey shading indicates isolates of the target subspecies for a given assay. C_T values are the average of two technical replicates, '-' indicates no amplification (C_T value recorded as 40.0). *NCPBPB; National Collection of Plant Pathogenic Bacteria, LMG; (BCCM-LMG) Belgian Co-ordinated Collections of Micro-organisms. † indicates the assay selected for complete validation.

Species	Isolate *	Xff_1-Rv-a	Xff_1-Rv-b	Xff_2	Xff_3-Fw-a †	Xff_3-Fw-b	Xfmv_1	Xfmv_2	Xfmv_3 †	Xfp_1 †	Xfmo_1_Rva	Xfmo_1_Rvb	Xfmo_2	Xfmo_4	Xfmo_5_Fwa	Xfmo_5_Fwb	Xfmo_6	Xfmo_7_Rva †	Xfmo_7_Rvb	Xfs_1	Xfs_2_Rva	Xfs_2_Rvb	Xfs_3	Xfs_4_Fwa	Xfs_4_Fwb †
<i>X. f. subsp. fastidiosa</i>	LMG 17159	17.5	17.9	16.3	16.7	18.0																			
<i>X. f. subsp. fastidiosa</i>	LMG 15099	15.8	15.8	12.9	16.1	16.2																			
<i>X. f. subsp. fastidiosa</i>	NCPP B 4605	17.9	16.8	16.4	16.0	17.1																			
<i>X. f. subsp. multiplex</i>	LMG 9063	34.2	33.1	27.4	-	-	27.0	25.1	25.8																
<i>X. f. subsp. multiplex</i>	NCPP B 4604	-	-	-	-	-	18.2	17.3	18.1														22.5		
<i>X. f. subsp. pauca</i>	NCPP B 4595	-	-	-	-	-	-	30.4	-	16.9															

[illegible]

Table 4. Results of sensitivity testing and assay efficiency for the 5 subspecies specific real-time PCR assays and the Harper universal assay (when tested with *X. fastidiosus* subsp. *fastidiosus*). Data is average values from 3 independent dilution series of DNA (in water), each tested with three technical replicates. The limit of detection (LOD) was defined as the lowest quantity of DNA where 100% of replicates were positive (n=9).

Assay	Limit of detection	C _T with neat DNA	C _T at LOD	R ² value	Slope	Amplification efficiency
<i>X.f.</i> subsp. <i>fastidiosus</i>	124 fg	16.3	35.1	0.9997	-3.4854	0.94
<i>X.f.</i> subsp. <i>multiplex</i>	182 fg	16.3	34.4	1	-3.5346	0.92
<i>X.f.</i> subsp. <i>pauca</i>	84.2 fg	15.9	34.3	0.9999	-3.5022	0.93
<i>X.f.</i> subsp. <i>morus</i>	59.2 fg	17.5	34.8	0.9985	-3.3522	0.99
<i>X.f.</i> subsp. <i>sandyi</i>	908 fg	15.6	32.2	0.9998	-3.4142	0.96
Universal (Harper)	124 fg	14.4	31.4	0.9999	-3.3786	0.98

Table 5. Overall analysis of the subspecies specific real-time PCR assays and the Harper universal assay (when tested with *X. fastidiosa* subsp. *fastidiosa*) limit of detection. Three independent 10-fold dilution series were prepared in water from a single extract and tested on 3 different days by 1 user. Each dilution was tested in three technical replicates. The average C_T value from positive wells (any C_T value <40) and the number and percentage of positive replicates were calculated. Results are classified (shaded) as; no shading when 'positive' (all 3 replicates wells positive with C_T value <35; pale grey shading when 'tentative' (C_T value >35 or not all 3 replicate wells positive); dark grey shading when 'negative' (all 3 replicates with C_T value >40).

Dilution	DNA per reaction	Day 1			Day 2			Day 3			Average		% positive replicates	
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	C _T	Number positive		
X. f. subsp. fastidiosa assay														
Neat DNA	12.42 ng	16.1	16.4	16.1	16.2	16.5	16.2	16.2	16.2	16.6	16.3	16.3	9	100.0
10 ⁻¹	1242 pg	20.0	20.2	19.9	19.8	20.3	19.6	19.8	19.8	20.4	19.7	20.0	9	100.0
10 ⁻²	124.2 pg	23.8	23.8	23.8	23.0	23.2	23.0	23.2	23.2	23.3	23.0	23.3	9	100.0
10 ⁻³	12.42 pg	27.2	27.5	27.2	26.4	26.7	26.4	26.5	26.5	26.7	26.3	26.8	9	100.0
10 ⁻⁴	1.242 pg	32.1	32.1	32.5	30.0	30.2	30.0	30.7	31.0	31.0	30.4	31.0	9	100.0
10 ⁻⁵	124 fg	37.9	38.2	38.3	33.6	33.3	32.7	34.3	34.0	34.0	34.0	35.1	9	100.0
10 ⁻⁶	12.4 fg	40.0	40.0	40.0	36.7	38.2	35.8	36.6	37.3	37.0	37.0	37.9	6	66.7
10 ⁻⁷	1.24 fg	40.0	40.0	40.0	40.0	40.0	38.5	40.0	40.0	40.0	38.7	39.7	2	22.2
10 ⁻⁸	124 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0
Negative control	0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0
X. f. subsp. multiplex assay														

[illegible]

X. f. subsp. morus assay

Neat DNA	5.92 ng	16.4	17.6	17.2	18.0	17.9	17.8	17.6	17.5	17.5	17.5	9	100.0
10 ⁻¹	592 pg	20.0	20.6	20.6	21.1	21.4	21.0	20.7	20.7	20.7	20.8	9	100.0
10 ⁻²	59.2 pg	24.2	25.0	24.9	24.9	24.8	24.6	24.2	24.4	24.3	24.6	9	100.0
10 ⁻³	5.92 pg	27.8	28.4	28.3	28.6	28.5	28.4	28.2	28.1	28.2	28.3	9	100.0
10 ⁻⁴	592 fg	31.6	32.4	32.2	32.1	32.0	32.3	31.8	31.6	31.4	31.9	9	100.0
10 ⁻⁵	59.2 fg	33.9	35.0	36.0	34.8	34.8	34.9	34.4	34.6	34.6	34.8	9	100.0
10 ⁻⁶	5.92 fg	40.0	40.0	40.0	40.0	39.3	40.0	37.8	38.8	40.0	39.5	3	33.3
10 ⁻⁷	592 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0
10 ⁻⁸	59.2 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0
Negative control	0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0

X. f. subsp. sandyi assay

Neat DNA	9.08 ng	15.6	15.4	15.4	15.6	15.7	15.6	15.7	15.7	15.8	15.6	9	100.0
10 ⁻¹	908 pg	19.1	19.0	19.3	19.0	19.0	19.1	18.8	18.8	18.9	19.0	9	100.0
10 ⁻²	90.8 pg	23.2	23.2	23.1	22.4	22.4	22.3	22.2	22.0	22.1	22.5	9	100.0
10 ⁻³	9.08 pg	26.5	26.4	26.5	25.6	25.6	25.3	25.5	25.5	25.6	25.8	9	100.0
10 ⁻⁴	908 fg	31.0	31.0	31.0	35.4	39.1	35.9	29.1	28.4	28.8	32.2	9	100.0
10 ⁻⁵	90.8 fg	34.4	34.5	34.1	38.2	40.0	38.8	32.4	32.0	32.0	35.2	8	88.9
10 ⁻⁶	9.08 fg	37.7	40.0	37.8	40.0	40.0	37.5	37.6	40.0	36.3	38.6	5	55.6
10 ⁻⁷	908 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	37.5	39.7	1	11.1
10 ⁻⁸	90.8 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0

Negative control	0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0	0.0
Universal assay (Harper <i>et al.</i>, 2010) with <i>X. f. subsp. fastidiosa</i>												
Neat DNA	12.42 ng	14.2	14.5	14.5	14.5	14.6	14.5	14.1	14.5	14.0	14.4	100.0
10 ⁻¹	1242 pg	18.1	18.0	18.0	17.5	17.5	17.3	17.5	17.4	17.5	17.6	100.0
10 ⁻²	124.2 pg	21.4	21.5	21.4	20.5	20.5	20.8	20.7	21.2	20.8	21.0	100.0
10 ⁻³	12.42 pg	24.9	25.1	24.7	24.2	24.3	24.3	24.0	24.3	24.6	24.5	100.0
10 ⁻⁴	1.242 pg	28.1	28.3	28.1	27.6	27.6	27.9	27.6	27.6	27.7	27.8	100.0
10 ⁻⁵	124 fg	31.5	31.6	31.2	31.3	31.2	31.0	31.7	31.7	31.1	31.4	100.0
10 ⁻⁶	12.4 fg	35.5	36.3	34.4	33.3	33.8	32.8	34.3	35.8	40.0	34.5	88.9
10 ⁻⁷	1.24 fg	40.0	37.1	36.3	36.7	35.4	36.9	40.0	37.4	36.5	36.6	77.8
10 ⁻⁸	124 ag	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0.0
Negative control	0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0.0

Table 6. Analysis of the Harper *et al.*, (2010) universal *X. fastidiosa* real-time PCR assay sensitivity with all of the *X. fastidiosa* subspecies. Dilution series (10-fold) were prepared in water and tested in three technical replicates. The limit of detection (LOD) was defined as the lowest quantity of DNA where 100% of replicates were classified as positive (n=3).

Subspecies	Isolate	Limit of detection	Dilution of LOD	C _T with neat DNA	C _T at LOD
<i>X.f. subsp. fastidiosa</i>	NCPPB4605	124 fg	10 ⁻⁵	14.2	31.5
<i>X.f. subsp. multiplex</i>	NCPPB4604	1.82 pg	10 ⁻⁴	16.9	32.1
<i>X.f. subsp. pauca</i>	NCPPB4595	842 fg	10 ⁻⁴	17.2	32.4
<i>X.f. subsp. morus</i>	NCPPB4589	59.2 fg	10 ⁻⁵	15.8	36.7
<i>X.f. subsp. sandyi</i>	NCPPB4606	9.08 fg	10 ⁻⁶	15.2	35.7

Table 7. Data on the validation of the subspecies specific real-time PCR assays according to the EPPO standard PM 7/98(2). Methods and results apply to all 5 developed assays.

Performance criteria	Result	Method used
Analytical sensitivity	Femtograms DNA detected	3 experiments on different days by one user. DNA extracted from pure culture was serially diluted through eight log dilutions in water and tested in 3 technical replicates (see Table 4 and Table 5)
Analytical specificity	100% accurate	1-3 targets, 4 other <i>X. fastidiosa</i> subspecies, 38 non-target species (50 isolates), 1 insect species (2 specimens), and 13 plant species analysed (see Table 1)
Selectivity	No observable impact of host plant	<i>X. fastidiosa</i> was detected in 10 different plant genera (see Table 1)
Repeatability	High level; 100%.	8 replicates of DNA from pure culture at varying concentrations; neat DNA (high level), at the assay
	Low level; 100%.	LOD (low level) and beyond the assay LOD (minimal level) were analysed (see Table 8)
	Minimal level; assay dependent (0% - 62.5%).	
Reproducibility	High level; 100%.	Analyses were performed by 2 different operators on 4 different devices on 3 different days with
	Low level; 92% - 100%.	freshly prepared reaction mix (see Table 8). 8 replicates of DNA from pure culture at varying
	Minimal level; assay dependent (8% - 63%).	concentrations; neat DNA (high level), at the assay LOD (low level) and beyond the assay LOD
		(minimal level) were analysed.
Diagnostic sensitivity	100%	11 infected plant samples analysed (see Table 1)
Diagnostic specificity	100%	13 non-infected plant samples analysed (see Table 1)

Table 8. Full data from repeatability and reproducibility testing according to the EPPO standard PM 7/98(2). Eight technical replicates were tested for DNA concentrations obtained from pure cultures (neat), at (10⁻⁴ dilution), and beyond (10⁻⁵ dilution) the limit of detection for each assay. Four different machines were tested by two different users on three different days. Two machines of model 7900 were used indicated by the suffix 1 or 2. The left column shows the number of positive replicates. No shading demonstrates 100% of replicates produced positive results and shading indicates less than 100% of replicates produced positive results. The right column shows the average C_T value obtained (from positive replicates only), 'n/a' indicates all replicates were negative (C_T value 40). *1 indicates the percentage of positive wells out of all tested replicates (n= 48).

DNA		7900-1	7900-1	7900-1	7900-1	7900-2	Viia7	7500	%					
Dilution	per reaction	User 1	User 1	User 1	User 2	User 1	User 1	User 1	positive wells*1					
<i>X. f. subsp. fastidiosa</i> assay														
Neat	12.42 ng	8	16.1	8	16.0	8	15.7	8	16.3	8	16.5	8	13.1	100
10 ⁻⁴	1.242 pg	8	34.3	8	33.8	8	34.6	8	34.7	8	34.6	8	31.8	100
10 ⁻⁵	124 fg	4	38.8	0	n/a	3	38.6	3	39.2	1	39.6	3	35.7	29
<i>X. f. subsp. multiplex</i> assay														
Neat	18.2 ng	8	16.4	8	16.4	8	16.2	8	16.5	8	17.2	8	13.5	100
10 ⁻⁴	1.82 pg	8	33.8	8	33.8	8	34.2	8	34.4	8	37.2	8	31.0	100
10 ⁻⁵	182 fg	0	n/a	0	n/a	0	n/a	0	n/a	0	n/a	4	36.1	8
<i>X. f. subsp. pauca</i> assay														
Neat	8.42 ng	8	16.5	8	16.1	8	16.5	8	16.2	8	16.5	8	13.0	100
10 ⁻⁴	842 fg	8	34.3	8	34.1	8	34.1	8	34.5	8	34.7	8	31.4	100
10 ⁻⁵	8.42 fg	0	n/a	4	38.4	2	39.0	0	n/a	0	n/a	4	34.5	21

***X. f.* subsp. *sandyi* assay**

Neat	9.08 ng	8	15.9	8	15.8	8	16.1	8	16.0	8	16.3	8	13.6	100
10 ⁻⁴	908 fg	8	29.1	8	32.5	8	29.2	8	29.6	8	29.8	8	30.1	100
10 ⁻⁵	90.8 fg	4	36.3	6	37.2	2	38.0	6	38.3	2	39.0	5	36.0	52

***X. f.* subsp. *morus* assay**

Neat	5.92 ng	8	17.7	8	17.5	8	17.5	8	17.7	8	17.9	8	15.4	100
10 ⁻⁴	592 fg	8	34.4	8	34.0	8	36.2	8	35.1	4	36.9	8	32.9	92
10 ⁻⁵	59.2 fg	5	35.5	6	37.2	6	38.7	6	38.3	0	n/a	7	35.0	63

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	Day 1			Day 2		Day 3	Day 4	
Identification using qPCR assays	DNA extraction	Universal qPCR	A	Subspecies qPCR x5	A			
	0.5 - 1 d	2.5 h	0.5 h	2.5 h	0.5 h			
Identification using MLST analysis	DNA extraction	Universal qPCR	A	MLST x7 genes; PCR, gel analysis, PCR clean-up, send for sequencing		Sanger sequencing	Sequence preparation	MLST analysis
	0.5 - 1 d	2.5 h	0.5 h	7 h		24 h	1 h	1 h

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