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ORIGINAL RESEARCH ARTICLE

Development and validation of X-*ComEC* qPCR, a novel assay for accurate universal detection of both *Xylella fastidiosa* and *Xylella taiwanensis*

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Abstract

Xylella fastidiosa is a devastating plant pathogenic bacteria known for its broad host range, in contrast to the related species *Xylella taiwanensis*, which is only known to cause disease in Asian pears. Despite the potential threats they pose to Australian agriculture, diagnostic assays capable of detecting both *Xylella* species are scarce. Bridging this critical gap, this study presents the development of the X-*ComEC* qPCR assay that targets a genus-specific DNA sequence, enabling accurate generic detection of all *Xylella* species. Benchmarking this novel qPCR assay against other published *Xylella* qPCR assays demonstrated its superior performance. The X-*ComEC* qPCR assay stands out as the only assay that can accurately detect both *X. fastidiosa* and *X. taiwanensis* without cross-reactivity with related bacteria. We have also carried out a comprehensive inter-laboratory test performance study, which demonstrated that the X-*ComEC* qPCR and the qPCR described by Harper et al. (Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications; erratum 2013) are highly robust and ready to use in Australia. Combining these two assays into a duplex qPCR enables simultaneous detection and species-level identification of *X. fastidiosa* and *X. taiwanensis.* The findings of this study have been incorporated into the Australian National Diagnostic Protocol for *Xylella* detection, arming diagnostic laboratories with critical knowledge to combat these globally significant pathogens. Overall, the collaborative and systematic approach employed in this study provides a model for developing and validating assays for all plant pathogens.

Keywords Molecular diagnostics · *Xylella* · Bacterial lead scorch · Pierce's disease · TaqMan real-time qPCR

Introduction

Xylella spp. are vector-borne, xylem-limited plant pathogenic bacteria. They cause severe, incurable disease in a variety of agriculturally important plants including Pierce's disease in grapevines, olive quick decline and almond leaf scorch (Hopkins and Purcell 2002; Rapicavoli et al. 2018). *Xylella* is transmitted by sharpshooters (Cicadellinae) and spittlebugs (Aphrophorinae), two widespread group of xylem-feeding insects (Krugner et al. 2019). Notably, glassy-winged sharpshooter (*Homalodisca vitripennis*) and meadow spittlebug (*Philaenus spumarius*) were main *Xylella* vectors responsible to the spreading of *Xylella* in North and South America, and in Southern Europe (Martelli et al. 2016). *Xylella* colonises the xylem vessel and

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obstructs water and nutrient transportation within the vessel, leading to host death (IPPC 2018). The *Xylella* genus consists of two species; *Xylella fastidiosa* (Xf) and *Xylella taiwanensis* (Xt; Wells et al. 1987; Su et al. 2016). Xf is subdivided into three main subspecies, *fastidiosa*, *pauca* and *multiplex* (Marcelletti and Scortichini 2016; Denancé et al. 2019). *Xylella* spp. are devastating plant pathogens of global concern. Since 2013, there have been several introductions into Western Europe of all three subspecies of Xf leading to devastating production losses on economically important crops (Rapicavoli et al. 2018). Different Xf subspecies have varying host ranges, with the total number of plant hosts amounting to over 700 species according to the latest figure (EFSA et al. 2024). In contrast, Nashi pears (*Pyrus pyrifolia*) are currently the only known host of Xt and there are no reports of Xt infecting other plant species (EFSA et al., 2023). Although Xt appears to have a limited host range and is only known to occur in Taiwan so far (Su et al. 2016), potential risks associated with Xt should not be overlooked. In Europe, the Panel of Plant Health of the European Food Safety Authority (EFSA) speculated that pear species other than Nashi pears are likely to also be susceptible to Xt and they have officially recognized Xt as a potential quarantine pest (EFSA et al., 2023).

Xylella has not been reported in Australia but Xf outbreaks in Europe have illustrated the substantial threat that Xf could pose to Australian native flora and crops. Many high value Australian commodities, such as olive, citrus, avocado, grape, stone fruit and almond, are susceptible to Xf (Hafi et al. 2021). Australian native species including *Hakea petiolaris* Meisn., *Grevillea alpina* Lindl., *Leptospermum laevigatum* (Gaertn.) F.Muell and *Swainsona galegifolia* (Andrews) R.Br. were found to be susceptible to Xf infection in a Californian study (Rathé et al. 2012a). The climate conditions in parts of Australia are similar to many of the *Xylella*-affected European regions such as southern Italy, France and Spain. Bioclimatic modelling based on current Xf distribution forecasted that temperate and tropical regions, including most parts of Australia, have a suitable climate for Xf to thrive, and the risk of Xf outbreaks may increase with climate change (Godefroid et al. 2022). While known *Xylella* vectors are not reportedly present in Australia, spittlebug species endemic to Australia such as *Bathyllus albicinctus* and *Philagra para* may vector *Xylella*, and contribute to spreading of the pathogen should it enter Australia (Rathé et al. 2012b; Martoni et al. 2024). If *Xylella* enters and establishes in Australia, it will severely impact Australian horticultural industries; predicted to cost 1.2 to 8.9 billion Australian dollars over 50 years (Hafi et al. 2021). Given the significant environmental and agricultural threat posed by *Xylella*, this pathogen is ranked the top priority plant pest by Australian plant biosecurity authorities (Department of Agriculture, Fisheries and Forestry, 2019).

Early detection is the key for timely intervention if *Xylella* is introduced into Australia; to prevent spread and increase the likelihood of eradication. However, *Xylella* infection is challenging to detect as infected plants could remain asymptomatic for an extended period of time which varies between hosts (IPPC 2018; EPPO 2023). Typical symptoms such as wilting, dieback and leaf scorch, can be mistaken for a myriad of other disorders, leading to diagnostic delays. *Xylella* is also difficult to isolate, as it is slow growing and requires specific culture media (Wells et al. 1987). Therefore, molecular diagnostic methods are critical tools for detection. Probe-based real-time quantitative PCR (qPCR) assays are commonly adopted for *Xylella* detection because they are fast, cost-effective and capable of direct detection in extracts from infected host plants or insect vectors. Particularly in an emergency response setting that demands rapid turnaround times, qPCR is preferred over conventional PCR because of its efficiency; detecting pathogens in real time without the need to run gel electrophoresis afterwards for visualization. Probe-based qPCR assays for Xf detection, such as those developed by Harper et al. (2010; erratum 2013), Francis et al. (2006), and Ouyang et al. (2013), were recommended by the European and Mediterranean Plant Protection Organization in their standard diagnostic protocols (EPPO 2023) and the International Plant Protection Convention (IPPC 2018) and are routinely used in plant diagnostic laboratories worldwide. On the contrary, qPCR assays with Xt detection capability were limited in numbers as Xt was only recognized as a new *Xylella* species in 2016 (Su et al. 2016). To our knowledge, there was only one qPCR assay that was designed to target Xt specifically (Su et al. 2023) and two generic qPCR assays for detection of both Xf and Xt (Ito and Suzaki 2017; Ito and Chiaki 2021).

Published qPCR assays developed for *Xylella* detection have not been robustly tested in Australia where *Xylella* has not yet been reported. As critical decision-making relies heavily upon the results generated by *Xylella* qPCR assays, they must be accurate and perform reliably and consistently across different diagnostic laboratories to prevent misdiagnosis. Hence, validation studies are essential to comprehensively evaluate and compare the performance of available *Xylella* qPCR assays under Australian conditions.

To improve the preparedness of Australian agricultural and horticultural industries to the threat posed by *Xylella*, an updated national diagnostic protocol (NDP) for both *Xylella* species was developed through the collaborative efforts of five national plant diagnostic laboratories across Australia and New Zealand. During the protocol preparation, we designed the X-*ComEC* qPCR primer and probe set for

detecting both *Xylella* species or detecting Xt specifically. Alongside this new qPCR assay, seven published *Xylella* qPCR assays were selected and comprehensively tested for their specificity, sensitivity, and reproducibility through an inter-laboratory test performance study (TPS; also known as ring tests) to determine which assays meet the needs of a compatible *Xylella* qPCR assay for Australian conditions. A duplex qPCR assay to detect and differentiate Xf and Xt was also developed and incorporated into the NDP. The findings of the qPCR assay development and the results of the TPS are reported in this study.

Materials and methods

Primer designs and analysis

As part of a larger project, we previously developed an Endpoint PCR assay for detection of *Xylella* spp. which targets the *ComEC/Rec2* gene region (Wong-Bajracharya et al., 2024). An additional qPCR reverse primer qPCR-X-*ComEC*-R was designed for this study to pair with the forward primer from the Endpoint PCR to produce a 156 bp amplicon from the *ComEC* gene region (Fig. 1). The qPCR amplicons were confirmed to be specific to Xf and Xt by BLASTn search against the NCBI nucleotide collection (nr/nt). Aside from the qPCR reverse primer, we designed two probes within the qPCR amplicon: the generic probe

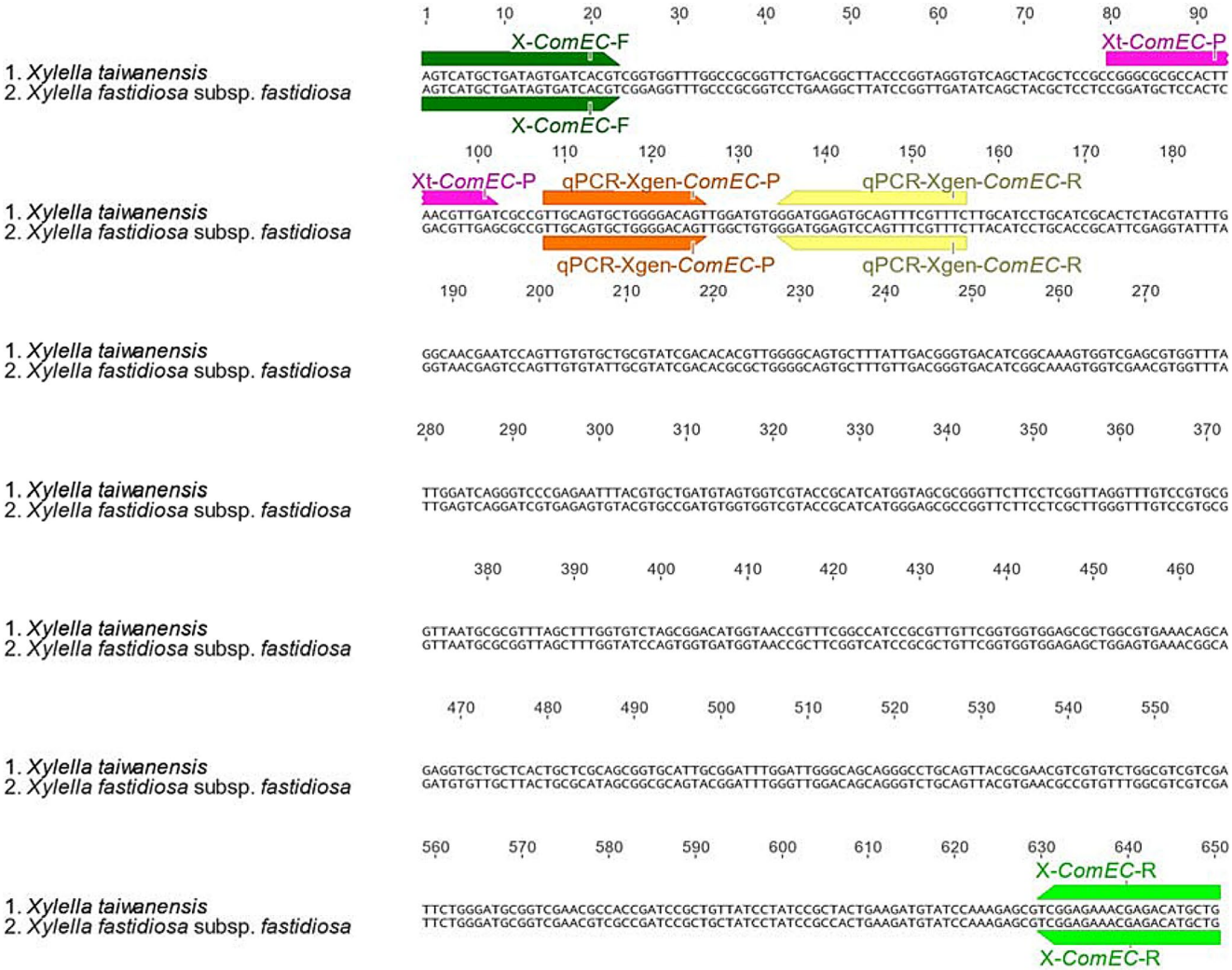


Fig. 1 Primer and probe design for the X-*ComEC* qPCR assay within the amplicon regions of X-*ComEC* PCR on both *Xylella* species. The newly designed reverse primer qPCR-Xgen-*ComEC*-R (yellow) was paired with the forward primer X-ComEC-F (dark green) we previously described in Wong-Bajracharya et al. (2024) to amplify a 156-base pair-long sequence. The probe Xt-*ComEC*-P (pink) can bind to DNA of *Xylella taiwanensis* but not *Xylella fastidiosa* subsp. *fastidiosa*, whereas the generic probe qPCR-Xgen-*ComEC*-P (orange) can bind to DNA of both species

qPCR-Xgen-*ComEC*-P and the Xt probe Xt-*ComEC*-P. The generic probe targeted both Xf and Xt DNA while the Xt probe was specific to Xt.

All primer sets included in this study were searched against a Xt representative genome (retrieved from RefSeq database, Accession = NZ_CP053627) to identify potential binding sites on Xt genomes using Primer3 (version 2.3.7) on Geneious Prime (version 2023.0.4). Up to two mismatches were allowed in the binding region during the search. Binding was only considered valid when both primers bind to the genome within a 1,000 bp region.

DNA testing panel

Forty-five DNA extracts sourced from bacterial monocultures were used to evaluate the specificity of the qPCR assays. Twenty-one *Xylella* isolates were retrieved from the National Collection of Plant Pathogenic Bacteria (NCPPB, United Kingdom), the Collection for Plant-associated Bacteria (CFBP, France) and the International Collection of Microorganisms from Plants (ICMP, New Zealand). These isolates represented diverse *Xylella* lineages including *X. fastidiosa* subsp. *fastidiosa* (Xff), *X. fastidiosa* subsp. *multiplex* (Xfm), *X. fastidiosa* subsp. *pauca* (Xfp), *X. fastidiosa* subsp. *sandyi*, and the type strain of Xt NCPPB4612 (also known as PLS229) that were originally isolated from various plant hosts (full list of all bacterial isolates are detailed in Supplementary Table S1). Non-target bacterial isolates were also included in our testing. Twenty-four Australian isolates of *Xanthomonas* and *Stenotrophomonas* (Supplementary Table S1), two genera of closely related bacteria, were sourced from the New South Wales Plant Pathology & Mycology Herbarium (Orange, Australia). DNA was extracted from these bacterial isolates using methods described previously in Wong-Bajracharya et al. (2024). Also included in the testing panel was DNA extracted directly from *Xylella*-infected host plants (16) and insect vectors (6) as described previously in Wong-Bajracharya et al., (2024).

Reaction mixtures and conditions for simplex *Xylella* qPCR assays

Eight simplex *Xylella* qPCR assays, including seven current qPCR assays described in previous publications and the X-*ComEC* qPCR developed in this study, were selected for evaluation. The primer and probe sets are summarised in Table 1. Apart from the Xt-*ComEC*-P probe, all probes used in this study contained a 5' Fluorescein (FAM) fluorophore, 3' Iowa Black FQ (iBFQ) quencher and an internal ZEN quencher (Integrated DNA Technologies, United States), which in some cases differed from the fluorophore and quencher described in the original studies. This decision was made to compare qPCR assay performance based on primer and probe design rather than probe chemistry. All qPCR reactions were performed in triplicate on a QuantStudio 5 Real-Time PCR System thermocycler (Applied Biosystem, United States). A no-template control sample served as a negative control.

The X-*ComEC* generic qPCR was prepared in a 20 µl reaction mixture with 300 nM forward primer X-*ComEC*-F, 300 nM reverse primer qPCR-Xgen-*ComEC*-R, 200 nM generic probe Xgen-*ComEC*-P, 1 mM dNTPs (Meridian Bioscience, United States), 6 mM $MgCl_2$, 2 µL 10X Immobuffer (Meridian Bioscience, United States), 0.4 µL 5 U/µL Immolase (Meridian Bioscience, United States) and 2 µl DNA template. The reaction was performed with the following conditions: pre-incubation at 50ºC for 2 min, initial denaturation at 95ºC for 2 min, followed by 40 cycles of 95ºC denaturation for 30 s and 64ºC annealing for 30 s.

Immolase is generally known to be a PCR DNA polymerase but it is also compatible for probe-based qPCR (Veldhoen et al. 2016). Immolase was chosen due to reagent accessibility issues at the time of assay development. To keep consistency, immolase was used in all internal testing. Compatibility with commercial qPCR reagents were validated in the inter-laboratory TPS as described below.

The reaction mixtures were prepared for the seven published qPCR assays (Table 1), using the primer and probe concentrations described in their respective publications, with Immolase (Meridian Bioscience, United States) and its associated reagents substituted for the reaction polymerase. An equal volume of DNA template (2 µl) was used for each reaction. The reaction was performed with reaction conditions and cycle numbers as described in their respective publications.

Comparative performance evaluation of qPCR analytical specificity and diagnostic sensitivity

Using the reaction conditions and mixtures described above, the analytical specificity and diagnostic sensitivity of the selected *Xylella* qPCR assays were comparatively validated in a multi-phasic manner using three different testing panels.

(1) Exclusivity test (analytical specificity)– a panel of DNA extracts from non-target bacteria was used to test the analytical specificity of assays. To check for cross-reactivities or false positive detections, the exclusivity panel included DNA samples of 24 Australian isolates of *Xanthomonas* and *Stenotrophomonas* and isolates of Xf (ICMP8731) and Xt (NCPPB4612) as positive controls (Supplementary Table S1). Assays that produced no false-positive detections of any of the non-target isolates advanced to the inclusivity test (2).

Table 1 Primers and probe sets used in the current and new qPCR assays described in this study. F = Forward primer; r = reverse primer; p = probe

Assay	Primer name (type)	Primer sequence (5' to 3')	Reference	Target gene	Amplicon length (bp)
Harper Xf qPCR	Harper XF-F (F)	CACGGCTGGTAACGGAAGA	Harper et al. (2010; erratum 2013)	16 S rRNA processing protein *rimM*	70
	Harper XF-R (R)	GGGTTGCGTGGTGAAATCAAG			
	Harper XF-P (P)	FAM-TCGCATCCCGTGGCTCAGTCC-iBFQ			
Ouyang Xf qPCR	Xf.Csp6F (F)	CCCATTACGCTTCAACCATT	Ouyang et al. (2013)	cobalamin synthesis protein	93
	Xf.Csp6R (R)	CCCAATCCATACGACTTGCT			
	Xf.Csp6P (P)	FAM-GGTGTGATTCGCAGCAAGGGC-iBFQ			
Dupas Xf qPCR	Dupas XF-F (F)	AACCTGCGTGACTCTGGTTT	Dupas et al. (2019)	ketol-acid reductoisomerase	118
	Dupas XF-R (R)	CATGTTTCGCTGCTTGGTCC			
	Dupas XF-P (P)	FAM-GCTCAGGCTGACGGTTTCACAGTGCA-iBFQ			
Agiletti Xf qPCR	Xf_Fw (F)	CGGGTACCGAGTCCATGTTG	Agiletti et al. (2019)	ribosome maturation factor *rimM*	60
	Xf_Rev (R)	CAATCAAACGCTTGCCAGTCT			
	Xf_Pr (P)	FAM-TGGTGCCCGTGGCTA-iBFQ			
Francis Xf qPCR	HL5 (F)	AAGGCAATAAACGCGCACTA	Francis et al. (2006)	hypothetical protein	221
	HL6 (R)	GGTTTTGCTGACTGGCAACA			
	HLP (P)	FAM-TGGCAGGCAGCAACGATACGGCT-iBFQ			
Ito & Suzaki generic qPCR	XrDf1 (F)	GGCTCATCCAATCGCACAA	Ito and Suzaki (2017)	16 S rRNA	172
	D-XrDr2 (R)	CGGACGGCAGCACAITGGTAIIIIIACCATGG			
	XrD-Pf (P)	FAM-CCTAAGGTCCCCTGCTT-iBFQ			
Ito & Chiaki generic qPCR	XrDf1 (F)	GGCTCATCCAATCGCACAA	Ito and Chiaki (2021)	16 S rRNA	172
	XLr4 (R)	CGGACGGCAGCACRKTGGT			
	XrD-Pf (P)	FAM-CCTAAGGTCCCCTGCTT-iBFQ			
X-*ComEC* generic qPCR	X-*ComEC*-F (F)	AGTCATGCTGATAGTGATCACGT	Wong-Bajracharya et al. (2024)	natural transformation protein *ComEC/RecA*	156
	qPCR-Xgen-*ComEC*-R (R)	CAGCATGTCTCGTTTCTCCGA	This study		
	qPCR-Xgen-*ComEC*-P(P)	FAM-TTGCAGTGCTGGGGACAGT-iBFQ			
Xf (Harper) / Xt (X-*ComEC*) duplex qPCR	X-*ComEC*-F (F)	AGTCATGCTGATAGTGATCACGT	Wong-Bajracharya et al. (2024)	natural transformation protein *ComEC*	156
	qPCR-Xgen-*ComEC*-R (R)	CAGCATGTCTCGTTTCTCCGA	This study		
	qPCR-Xt-*ComEC*-P (P)	Cy5-CGGGCGCGCCACTTAACGTTGAT-iBRQ			
	Harper XF-F (F)	CACGGCTGGTAACGGAAGA	Harper et al. (2010; erratum 2013)	16 S rRNA processing protein *rimM*	70
	Harper XF-R (R)	GGGTTGCGTGGTGAAATCAAG			
	Harper XF-P (P)	FAM-TCGCATCCCGTGGCTCAGTCC-iBFQ			

(2) Inclusivity test (analytical specificity)– a panel of DNA extracted from 19 Xf isolates of different subspecies was used to further evaluate analytical specificity of the selected assays in terms of true positive and false negative rates. This panel included nine isolates of subsp. *fastidiosa*, four isolates of subsp. *multiplex*, four isolates of subsp. *pauca* and two isolates of subsp. *sandyi* (Supplementary Table S1). Assays that produced true positive results for all Xf isolates proceeded to the host test

(3) Host test (diagnostic sensitivity)– DNA extracts that originated from *Xylella*-infected host plants and insect vectors were used to test the diagnostic sensitivity of each assay, which was defined as the percentage of true positive detection on all infected plants and insect samples. Four of the best performing assays were included in the inter-laboratory TPS to assess their analytical sensitivity and reproducibility.

Reaction mixtures and conditions for Xf (Harper) / Xt (X-*ComEC*) duplex qPCR

The Xf (Harper) / Xt (X-*ComEC*) duplex qPCR (referred to as the Xf/Xt duplex qPCR thereafter) contained primers and probes derived from this study and from the published study by Harper et al. (2010; erratum 2013). The 20 µl reaction mixture contained the following: 300 nM forward primer X-*ComEC*-F, 300 nM reverse primer qPCR-Xgen-*ComEC*-R, 200 nM Xt probe Xt-*ComEC*-P, 300 nM primer pairs XF-F/R, 200nM Xf probe XF-P, 1 mM dNTPs

(Meridian Bioscience, United States), 6 mM $MgCl_2$, 2 µL 10X Immobuffer (Meridian Bioscience, United States), 0.4 µL 5 U/µL Immolase (Meridian Bioscience, United States) and 2 µl DNA template. The qPCR was performed with the following reaction conditions: pre-incubation at 50ºC for 2 min, initial denaturation at 95ºC for 2 min, followed by 40 cycles of 95ºC denaturation for 10 s and 62ºC annealing for 40 s. The Xt probe was detected using the Cy5 channel and the Xf probe was detected using the FAM channel. The analytical specificity of the Xf / Xt duplex qPCR was evaluated using the two testing panels (exclusivity and inclusivity) as described above. The difference in Cq value of the Harper XF-P probe was calculated by subtracting the mean Cq value in the duplex qPCR format with the mean Cq value in the simplex qPCR format. An unpaired, two-sample, two-tailed t-test was performed to determine whether there was a significant difference in the Cq values obtained in the duplex and simplex format.

Inter-laboratory test performance study on analytical sensitivity and reproducibility

To evaluate the analytical sensitivity and reproducibility of four selected simplex qPCR assays (Harper Xf qPCR, Ouyang Xf qPCR, Dupas Xf qPCR and X-*ComEC* generic qPCR) and the Xf/Xt duplex qPCR assay, molecular biologists from five national diagnostic laboratories in Australia and New Zealand participated in an inter-laboratory TPS. An aliquot of 2.4 ng/µl DNA sample from each of four representative *Xylella* isolates; ICMP 8731 (Xff), ICMP 8739 (Xfm), CFBP 8072 (Xfp) and NCPPB 4612 (Xt), were provided to each laboratory. For each DNA sample, testing participants were instructed to prepare a serial dilution series from 0.24 to 2.4×10^{-10} ng/µl with 10-fold dilution at each step. The participants then tested the detection limit of the qPCR assays using the same concentration of primers and probes and volume of DNA template; with qPCR reagents and equipment listed in Supplementary Table S2. All participants used probes with the same fluorophore as original design (Table 1) in all cases except one. Participant B performed their testing on the qPCR-Xt-*ComEC*-P probe using a VIC fluorophore instead due to incompatibility of their qPCR machine with Cy5 fluorophore. The qPCRs were performed in triplicate and with the reaction conditions described previously. When amplifications were detected in all three replicates, the detection was considered valid. The amplification efficiency of the qPCR assays was tested for each DNA sample that had more than two valid detections along the dilution series using the method stated in Broeders et al. (2014). The intra-assay mean quantification cycle (Cq) values were taken by averaging the Cq values from the triplicates. To measure the variability between runs performed by different TPS participants, we also computed the inter-assay mean, standard deviation (SD) and coefficient of variation (CV) based on the intra-assay mean Cq values. These calculations were computed using R (version 4.1.2; R Core Team 2021) to assess the reproducibility of the qPCR assays across laboratories.

A follow-up validation has been carried out by an independent molecular biologist to verify Xt detection ability by these four selected assays. In this validation study, a new DNA sample was extracted from a fresh culture of the Xt strain NCPPB4612 and used for dilution series preparation, following the methods previously described. This new DNA extract was used to perform analytical sensitivity testing. Another freshly prepared DNA extract of the Xf strain ICMP8731 was used as a positive control. QuantStudio 5 and Immolase were the qPCR machine and reagent used in this independent testing.

Results

Comparative performance evaluation to select the best assay to detect both *Xylella* species

Comparative performance evaluation showed that the published assays and the X-*ComEC* generic qPCR assays developed in this study varied in their analytical specificity (Table 2). In the exclusivity test phase, six out of eight

Table 2 Analytical specificity performance of X-*ComEC* qPCR assay comparing to published qPCR assays on the exclusivity and inclusivity testing panel. Number of detection/total number of isolates in the testing panel were shown in brackets. N/A=not applicable

	True negative rate	False negative rate	True positive rate		False negative rate	
Testing bacteria	non-target bacteria		*X. fastidiosa*	*X. taiwanensis*	*X. fastidiosa*	*X. taiwanensis*
X-*ComEC* generic qPCR (This study)	100% (24/24)	0% (0/24)	100% (20/20)	100% (1/1)	0% (0/20)	0% (0/1)
Harper Xf qPCR	100% (24/24)	0% (0/24)	100% (20/20)	N/A	0% (0/20)	N/A
Ouyang Xf qPCR	100% (24/24)	0% (0/24)	100% (20/20)	N/A	0% (0/20)	N/A
Dupas Xf qPCR	100% (24/24)	0% (0/24)	100% (20/20)	N/A	0% (0/20)	N/A
Agiletti Xf qPCR	100% (24/24)	0% (0/24)	100% (20/20)	N/A	0% (0/20)	N/A
Francis Xf qPCR	100% (24/24)	0% (0/24)	100% (20/20)	N/A	0% (0/20)	N/A
Ito & Suzaki generic qPCR	75% (18/24)	25% (6/24)	100% (20/20)	100% (1/1)	0% (0/20)	0% (0/1)
Ito & Chiaki generic qPCR	79% (19/24)	21% (5/24)	100% (20/20)	100% (1/1)	0% (0/20)	0% (0/1)

assays, including the X-*ComEC* generic qPCR developed in this study, did not amplify non-target bacterial DNA from related genera of *Xanthomonas* or *Stenotrophomonas* (Supplementary Table S1). All eight assays successfully detected the positive control Xf isolate ICMP 8731. The X-*ComEC* generic qPCR and two other published generic qPCR assays (Ito & Chiaki qPCR and Ito & Suzaki qPCR) were designed to detect Xt and all three assays amplified DNA of the Xt isolate NCPPB4612 (Supplementary Table S1). Despite having the capability to detect both Xf and Xt, the Ito & Chiaki qPCR and Ito & Suzaki qPCR generated false positives with non-target *Stenotrophomonas* isolates from Australia and therefore were excluded from subsequent testing. All six assays subjected to the inclusivity test successfully amplified all Xf isolates included in the panel including Xf subspecies *fastidiosa*, *sandyi*, *multiplex* and *pauca* (Table 2).

All six assays were also assessed for their diagnostic sensitivity at the host test phase, where we found that they were able to detect Xf in all infected insect samples and most infected plant samples (Table 3). Harper Xf qPCR and X-*ComEC* generic qPCR assays has a diagnostic sensitivity of 100%, detecting positively to all infected samples in our panel. They were the only assays that returned positive results for one of the infected *Vitis vinifera* samples (Xf concit plant B1S1). The Cq value for this sample was above 30 cycles for both assays, indicating a lower bacterial titre than other samples. The other assays had diagnostic sensitivity ranging between 90% and 95%. Aglietti Xf qPCR was the only assay that could not detect Xf from the infected *Olea* sample Xf cocit plants B3S2.

In this study, the X-*ComEC* generic qPCR was the only assay that successfully detected both *Xylella* species and did not generate false positives with non-target species.

Table 3 Results of X-*ComEC* qPCR assay generated on plant and insect DNA extracts compared to those produced by published qPCR assays. Nd = not detected

			Assay	X-ComEC generic qPCR (This study)	Harper Xf qPCR	Ouyang Xf qPCR	Dupas Xf qPCR	Agiletti Xf qPCR	Francis Xf qPCR
Sample name	Host/vector	Species	Subspecies	Cq value					
Healthy vitis S4	*Vitis vinifera*	Not infected	Not applicable	nd	nd	nd	nd	nd	nd
Xf cocit plants B1S1	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	36.60	33.76	nd	nd	nd	nd
Xf cocit plants B1S2	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	26.03	26.22	27.24	22.06	22.32	23.41
Xf cocit plants B1S3	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	26.95	26.03	27.28	21.81	22.59	24.73
Xf cocit plants B1S4	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	27.81	27.82	29.41	23.50	23.04	24.66
Xf cocit plants B2S1	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	25.96	26.50	26.72	23.05	22.40	23.31
Xf cocit plants B2S2	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	25.77	25.95	26.05	20.67	23.42	23.62
Xf cocit plants B2S3	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	27.71	28.18	28.58	21.35	27.24	24.84
Xf cocit plants B2S4	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	27.10	27.73	27.63	24.00	25.44	25.62
Xf cocit plants B2S5	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	25.42	25.30	26.69	22.60	23.88	23.88
Xf cocit plants B2S6	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	23.84	24.39	25.48	20.35	22.15	30.87
Xf cocit plants B2S7	*Vitis vinifera*	*Xylella fastidiosa*	*fastidiosa*	31.99	33.27	34.83	28.32	37.98	21.43
Xf cocit plants B3S1	*Olea europea*	*Xylella fastidiosa*	*fastidiosa*	26.33	26.48	27.40	22.81	24.75	24.80
Xf cocit plants B3S2	*Olea europea*	*Xylella fastidiosa*	*fastidiosa*	32.81	31.86	33.81	28.70	nd	32.30
Xf cocit plants B4S2	*Olea europea*	*Xylella fastidiosa*	*fastidiosa*	27.72	26.88	29.85	23.75	25.37	24.87
Xf cocit plants B5S1	*Nerium oleander*	*Xylella fastidiosa*	*fastidiosa*	24.07	23.98	25.60	21.10	22.22	22.73
Xf-exposed insect-1	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	27.41	26.85	37.29	24.84	26.56	26.86
Xf-exposed insect-2	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	31.09	30.70	29.07	28.04	29.61	25.71
Xf-exposed insect-3	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	32.64	32.75	28.03	28.56	31.86	24.85
Xf-exposed insect-4	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	28.98	28.59	35.56	25.29	27.92	32.85
Xf-exposed insect-5	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	32.12	32.57	32.22	27.84	27.90	29.01
Xf-exposed insect-6	*Homalodisca citripennis*	*Xylella fastidiosa*	*multiplex*	32.14	35.53	32.02	27.69	32.51	29.81
			Diagnostic sensitivity	95.45%	95.45%	90.91%	90.91%	86.36%	90.91%

Inter-laboratory comparison of qPCR analytical sensitivity and reproducibility

Four of the best performing qPCR assays in all three phases of performance evaluation (the X-*ComEC* generic qPCR described in this study, Harper Xf qPCR, Ouyang Xf qPCR and Dupas Xf qPCR) were selected for further assessment of their analytical sensitivity and reproducibility by inter-laboratory TPS. Figure 2 shows the comparison of detection limits generated by the participants using different qPCR reagents and equipment. All participants reported the successful detection of all three Xf subspecies using every assay (Fig. 2). Typically, the limit of detection ranged from 2.4×10^{-3} to 2.4×10^{-5} ng/µl with a Cq value between 20 and 38 cycles. However, detections outside of this range were observed in some cases: participant C reported a higher limit of detection using the Ouyang Xf qPCR at 0.24 to 0.024 ng/µl, whereas participant B detected amplifications in only two out of three replicates using Ouyang Xf qPCR on five different concentrations. The X-*ComEC* generic qPCR result reported by the participant C had generally higher Cq values than other participants. These variability in results indicated some assays were more sensitive to changes in operators, equipment and reagents used.

Of the four PCR assays, only the X-*ComEC* generic qPCR assay was designed to detect both Xf and Xt (Fig. 2). All testing participants reported positive detections of Xt when using the X-*ComEC* generic qPCR with the detection limit ranging from 2.4×10^{-4} to as low as 2.4×10^{-6} ng/µl. Amplification of Xt DNA by the three published qPCR assays was unexpected because these assays were designed for Xf-specific detection. In silico primer analysis also identified no valid binding sites for these primer sets on the Xt genome. When using the three published qPCR assays, most testing participants reported no detection for Xt. However, non-specific amplifications of Xt DNA were noted in some

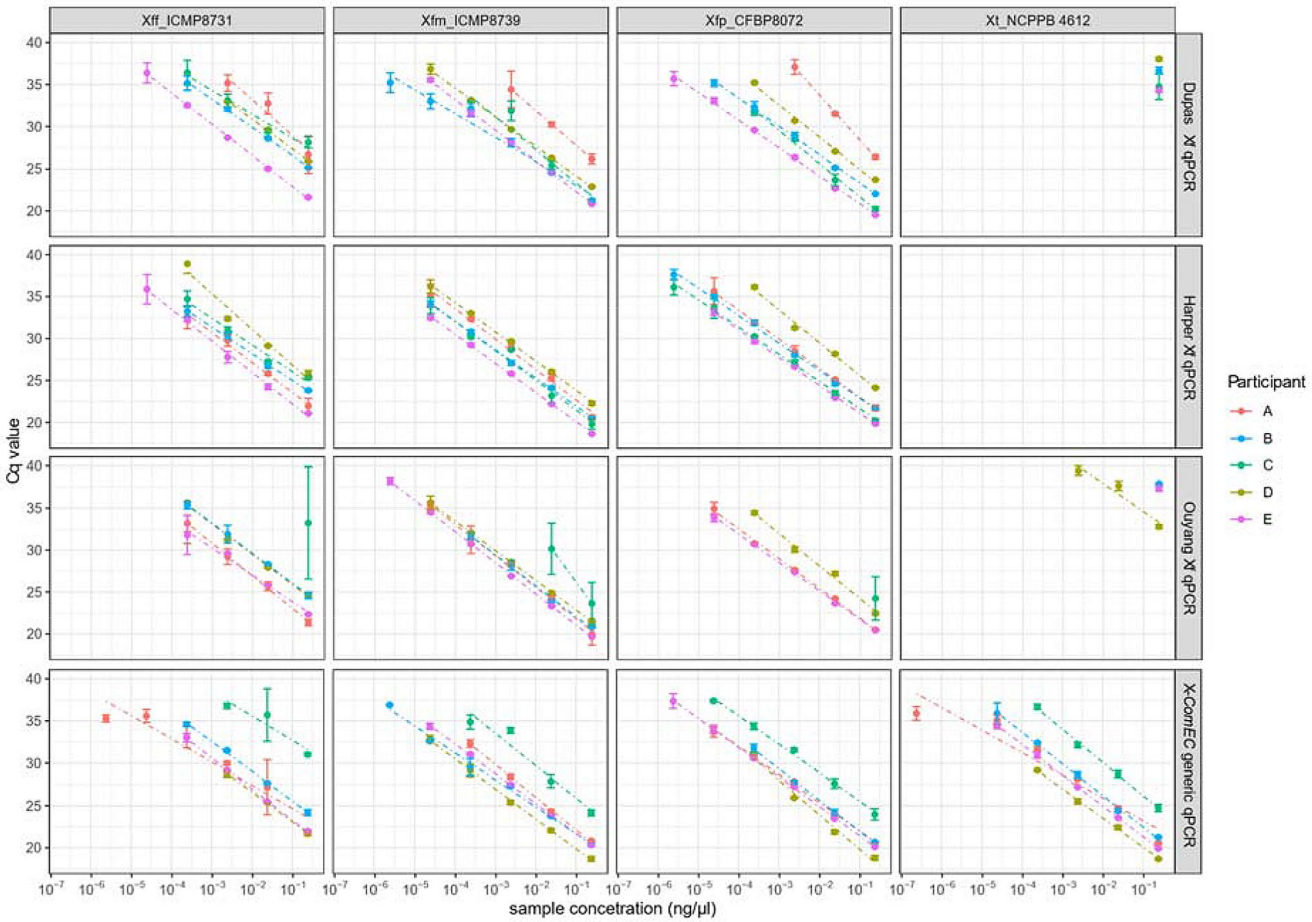
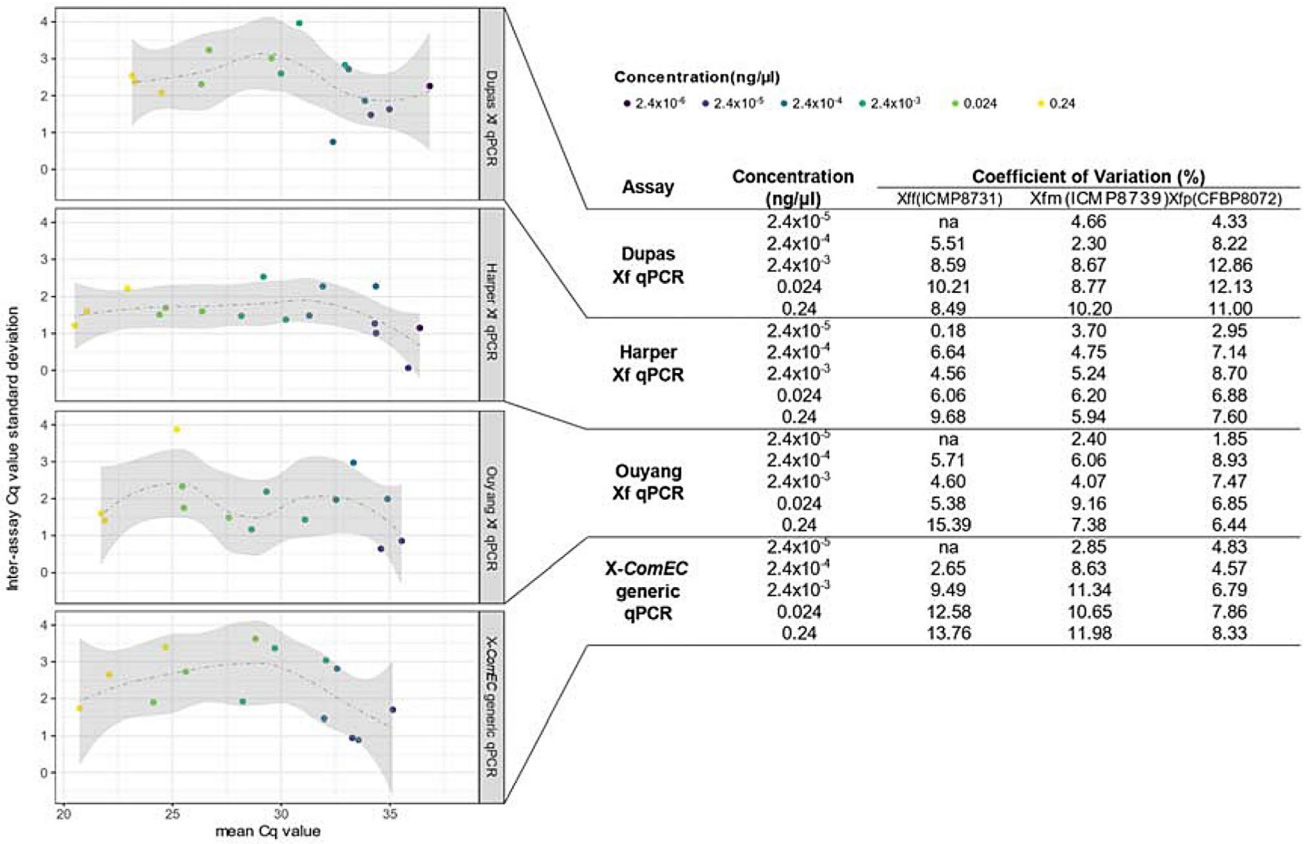


Fig. 2 Standard curve comparing the analytical sensitivity of qPCR assays (row) detection of four *Xylella* isolates (column) generated by independent runs performed by different test performance study participants (line colour). The isolates included in the testing are ICMP8731 (*X. fastidiosa* subsp. *fastidiosa*; Xff), ICMP8739 (*X. fastidiosa* subsp. *multiplex*; Xfm), CFBP8072 (*X. fastidiosa* subsp. *pauca*; Xfp) and NCPPB4612 (*X. taiwanensis*; Xt). The points represent the intra-assay mean quantification cycle (Cq) value calculated based on triplicate measurements, whereas the error bar denotes the intra-assay standard error. Only valid detections (amplification detected in all three replicates) were shown in this figure

instances, especially at the highest DNA template concentrations. Four participants observed amplification of Xt DNA at 0.24 ng/µl using the Dupas Xf qPCR, while three participants observed amplification using the Ouyang Xf qPCR.

Participant D reported non-specific amplification of Xt DNA at as low as 2.4×10^{-3} ng/µl concentration when using the Ouyang Xf qPCR. A commonality of these non-specific amplifications was a relatively high Cq value (34.31–38.05 for Dupas Xf qPCR and 32.80-39.46 for Ouyang Xf qPCR) which could be attributed to either PCR artifact formation at later amplification cycles or a low-level cross contamination. A follow-up validation was conducted by an independent testing participant using a newly extracted Xt DNA sample (Supplementary Table S5) and they reported no amplification of Xt DNA by neither of these three published Xf qPCR assays.

The amplification efficiencies of all assays on different samples were found to be 80–120% in most cases, within a desirable range for qualitative detections (Broeders et al. 2014; Supplementary Table S3). Harper Xf qPCR consistently performed across all testing participants in terms of Xf detection, as indicated by the closely aligned and uniform line clusters in the standard curve in Fig. 2. Among all the qPCR assays evaluated, the Harper Xf qPCR had the least fluctuation in its inter-assay standard deviation across the entire Cq range (Fig. 3). The Harper Xf qPCR assay also had the lowest inter-assay CV in its Cq measurement at an average of 5.6% across all sample types, maintaining a low inter-assay CV at less than 10% for all samples. The inter-assay Cq value for the different qPCR assays varied with a standard deviation of 1 to 4 cycles (Fig. 3). In all assays included in the TPS, samples of higher DNA concentrations tended to correlate with a higher CV. These variations could be associated with the differences in thermocyclers, techniques and/or reagents used by different participants. Overall, the Harper Xf qPCR was found to be the most reproducible assay for Xf detection.



Assay	Concentration (ng/µl)	Coefficient of Variation (%) Xff(ICMP8731)	Xfm (ICMP8739)	Xfp(CFBP8072)
Dupas Xf qPCR	$2.4x10^{-5}$	na	4.66	4.33
	$2.4x10^{-4}$	5.51	2.30	8.22
	$2.4x10^{-3}$	8.59	8.67	12.86
	0.024	10.21	8.77	12.13
	0.24	8.49	10.20	11.00
Harper Xf qPCR	$2.4x10^{-5}$	0.18	3.70	2.95
	$2.4x10^{-4}$	6.64	4.75	7.14
	$2.4x10^{-3}$	4.56	5.24	8.70
	0.024	6.06	6.20	6.88
	0.24	9.68	5.94	7.60
Ouyang Xf qPCR	$2.4x10^{-5}$	na	2.40	1.85
	$2.4x10^{-4}$	5.71	6.06	8.93
	$2.4x10^{-3}$	4.60	4.07	7.47
	0.024	5.38	9.16	6.85
	0.24	15.39	7.38	6.44
X-*ComEC* generic qPCR	$2.4x10^{-5}$	na	2.85	4.83
	$2.4x10^{-4}$	2.65	8.63	4.57
	$2.4x10^{-3}$	9.49	11.34	6.79
	0.024	12.58	10.65	7.86
	0.24	13.76	11.98	8.33

Fig. 3 Reproducibility of the four *Xylella* qPCR assays in the test performance study (TPS). The dot-plot on the left showed the inter-assay quantification cycle (Cq) value standard deviation (y-axis) and mean (x-axis) generated with the *Xylella fastidiosa* DNA dilution series based on the independent runs performed by different TPS participants. The color of the dots indicated the concentration of the template DNA. The dotted-line and the grey shade represented the predicted standard deviation across the Cq range and the confidence level based on local polynomial regression fitting. The table on the right summarise the coefficient of variation of the mean Cq measurements reported by different TPS participants. na = not available

Table 4 Results of analytical specificity testing of Xf (Harper) / Xt (X-*ComEC*) duplex qPCR in comparison to Harper Xf simplex qPCR. Results shown here were obtained from a single laboratory testing. N/A = not applicable; nd = not detected

	Assay	Xf (Harper) / Xt (X-ComEC) duplex qPCR		Harper Xf simplex qPCR	Inter-assay Cq difference (duplex - simplex)
	Probe	qPCR-Xt-ComEC-*P*	XF-*P*	XF-*P*	
Sample	Species	Mean Cq value			
DAR65801	*Stenotrophomonas maltophilia*	nd	nd	nd	N/A
DAR72045	*Stenotrophomonas maltophilia*	nd	nd	nd	N/A
DAR75512	*Stenotrophomonas maltophilia*	nd	nd	nd	N/A
DAR76132	*Stenotrophomonas maltophilia*	nd	nd	nd	N/A
DAR77232	*Stenotrophomonas maltophilia*	nd	nd	nd	N/A
DAR77233	*Stenotrophomonas* sp.	nd	nd	nd	N/A
DAR77234	*Stenotrophomonas* sp.	nd	nd	nd	N/A
DAR77236	*Stenotrophomonas* sp.	nd	nd	nd	N/A
DAR77237	*Stenotrophomonas* sp.	nd	nd	nd	N/A
1622 B Strain	*Xanthomonas fuscans*	nd	nd	nd	N/A
P03- 83	*Xanthomonas alfalfae*	nd	nd	nd	N/A
DAR41379	*Xanthomonas vasicola*	nd	nd	nd	N/A
DAR73877	*Xanthomonas vesicatoria*	nd	nd	nd	N/A
DAR35705	*Xanthomonas translucens*	nd	nd	nd	N/A
DAR49849	*Xanthomonas hortorum*	nd	nd	nd	N/A
DAR33337	*Xanthomonas arboricola*	nd	nd	nd	N/A
DAR82645	*Xanthomonas campestris*	nd	nd	nd	N/A
DAR82580	*Xanthomonas* sp.	nd	nd	nd	N/A
DAR82611	*Xanthomonas oryzae*	nd	nd	nd	N/A
DAR30526	*Xanthomonas campestris*	nd	nd	nd	N/A
VPRI41552	*Xanthomonas campestris*	nd	nd	nd	N/A
DAR82627	*Xanthomonas campestris*	nd	nd	nd	N/A
DAR82711	*Xanthomonas campestris*	nd	nd	nd	N/A
DAR 72,015	*Xanthomonas translucens*	nd	nd	nd	N/A
NCPPB4612	*Xylella taiwanensis*	22.59	nd	nd	N/A
ICMP8731	*Xylella fastidiosa*	nd	21.3	23.90	-2.58
ICMP8739	*Xylella fastidiosa*	nd	23.3	20.40	2.90
ICMP8740	*Xylella fastidiosa*	nd	18.6	23.47	-4.87
ICMP8742	*Xylella fastidiosa*	nd	22.1	23.28	-1.18
ICMP8745	*Xylella fastidiosa*	nd	27.0	27.28	-0.30
ICMP15197	*Xylella fastidiosa*	nd	26.8	26.64	0.12
NCPPB4604	*Xylella fastidiosa*	nd	19.5	18.46	1.00
CFBP8495	*Xylella fastidiosa*	nd	19.5	18.13	1.37
CFBP8071	*Xylella fastidiosa*	nd	17.9	18.54	-0.62
CFBP8524	*Xylella fastidiosa*	nd	16.7	15.64	1.06
CFBP8173	*Xylella fastidiosa*	nd	18.0	16.29	1.72
CFBP7969	*Xylella fastidiosa*	nd	20.5	19.33	1.16
CFBP7970	*Xylella fastidiosa*	nd	18.6	17.26	1.33
CFBP8477	*Xylella fastidiosa*	nd	19.4	18.21	1.16
CFBP8072	*Xylella fastidiosa*	nd	20.8	19.53	1.25
CFBP8073	*Xylella fastidiosa*	nd	18.5	17.06	1.47
CFBP8082	*Xylella fastidiosa*	nd	18.6	17.71	0.86
NCPPB4605	*Xylella fastidiosa*	nd	19.0	18.02	1.00
CFBP8077	*Xylella fastidiosa*	nd	18.1	17.24	0.88

Xf (Harper) / Xt (X-*ComEC*) duplex qPCR detects and differentiates *Xylella* species

To detect both *Xylella* species, we complemented the Harper Xf qPCR with the Xt-*ComEC* probe developed in this study (referred as the Xf/Xt duplex qPCR). We then assessed the duplex assay's analytical specificity internally, as well as its analytical sensitivity and reproducibility through the TPS.

As shown in Table 4, the Xf / Xt duplex qPCR assay had equivalent specificity towards Xf as the simplex Harper

Xf qPCR based on testing done by one laboratory. In most cases, the inter-assay difference in Cq value for Xf samples was less than two cycles. The Cq value for Xf samples in the duplex qPCR was not significantly different from that of the simplex qPCR (p-value > 0.1), indicating that the sensitivity of the Harper XF-P probe in the duplex reaction was comparable to the simplex counterpart. There was also no non-target detection of any of the *Xanthomonas* or *Stenotrophomonas* isolates in our exclusivity panel (Table 4). The Xt sample was detected using the Cy5 labelled Xt-*ComEC*-P probe in the Xf / Xt duplex qPCR assay. Therefore, the Xf / Xt duplex qPCR successfully detected and differentiated between Xf and Xt. The detection limit for all Xf samples was reported by four of five inter-laboratory TPS participants to range from 2.4×10^{-3} to 2.4×10^{-4} ng/µl (Supplementary Table S4), which is comparable to the Harper Xf simplex qPCR. Similarly, the detection limit for the Xt sample ranged from 2.4×10^{-3} to 2.4×10^{-4} ng/µl. Participant B reported a false positive detection by the Harper XF-P probe on the Xt isolate NCPPB4612 at a concentration of 2.4×10^{-3} ng/µl. While the exact reason of this unexpected false positive results could not be substantiated, this might be related to the switch from Cy5 to VIC as the reporter dye in Xt detection, which was implemented due to compatibility issues with their qPCR instruments. Aside from participant B, there were no reported non-specific detections of the Harper Xf probe or the Xt-*ComEC*-P probe.

Discussion

Xylella is ranked by the Australian government as the number one plant biosecurity threat to our agricultural and horticultural industries. Given both Xf and Xt are exotic to Australia, a generic qPCR assay to detect both *Xylella* species will be an important tool; strengthening Australia's diagnostic capability and facilitating a swift response in the event of an incursion. During our search for *Xylella* diagnostic methods suitable for incorporation into an Australian NDP, we evaluated the performance of current *Xylella* qPCR assays and introduced a brand new *Xylella* generic qPCR assay—the X-*ComEC* qPCR. Although a number of *Xylella* qPCR assays have been developed and are commonly used by plant diagnostic laboratories globally, their suitability for Xt detection was not known. This is particularly true for the older qPCR assays developed prior to the reclassification of Xt as a separate species (Su et al. 2016) or the publication of its genome (Weng et al. 2021). These include the Ouyang Xf qPCR, Harper Xf qPCR and Francis Xf qPCR. By comparing the specificity of X-*ComEC* qPCR with the five published Xf qPCR assays, the assay presented here was among the three qPCR assays compatible for detection of both Xt and Xf and differentiation of the two species. The X-*ComEC* qPCR was designed to target and amplify a genus-specific sequence (the *ComEC* gene) which we identified in our previous study through genomic comparison with related bacterial species (Wong-Bajracharya et al., 2024). The exclusivity test verified that the X-*ComEC* qPCR assay is highly specific and there was no non-target amplification of DNA from related bacterial species. In contrast, the other two *Xylella* generic qPCR assays targeting the 16 S rRNA hypervariable gene region had cross-reactivity with *Stenotrophomonas* spp. isolates found in Australia, rendering them unfit for *Xylella* detection in the region. Overall, in this study the X-*ComEC* qPCR assay was the most accurate assay for generic detection of both *Xylella* species.

Detecting *Xylella* can be challenging due to its fastidious nature and potential asymptomatic infections. Therefore, an effective qPCR assay must be sensitive enough for culture-independent detection. Our testing showed that the X-*ComEC* qPCR assay amplified Xf in DNA extracts from Xf-infected plants and insect vectors and performed at a similar level to three other published Xf qPCR assays; the Harper Xf qPCR, Ouyang Xf qPCR and Dupas Xf qPCR. While these published assays were reportedly sensitive, different samples, reagents and equipment were used in their associated studies, making it impossible to fairly compare their detection limits with the new assay. The inter-laboratory TPS conducted in this study addressed this knowledge gap by unbiasedly comparing the sensitivity and reproducibility of the new and published assays. The validation performed met the tier 3 validation guideline as described by Cardwell et al. (2018). All four assays successfully detected all three Xf subspecies included in our testing, but the limit of detection was reported at different levels by our TPS participants. Late amplifications of Xt DNA by Dupas and Ouyang Xf qPCR assays were also observed by some participants. These variations were expected and could be attributed to the differences in handling, reagents and equipment (Groth-Helms et al. 2023). However, an optimal assay for successful implementation as a diagnostic tool for *Xylella* diseases should be expected to perform consistently in variable laboratory conditions. Overall, our TPS results indicate that the Harper Xf qPCR assay exhibits the most robust performance and consistency, making it the recommended choice for Xf detection. This aligns with the EPPO's recommendation of the Harper Xf qPCR as the 'most commonly used' test in the EPPO region (EPPO 2023). However, the X-*ComEC* generic qPCR assay developed in this study can detect Xt as well as Xf. Therefore, the X-*ComEC* generic qPCR would be a preferrable assay if Xt is considered a potential pathogen (for instance, when testing a pear sample exhibiting leaf scorching symptoms).

Harnessing the strength of Harper Xf qPCR in robustness and that of X-*ComEC* qPCR in Xt compatibility, we combined the two assays into a duplex assay, namely the Xf (Harper) / Xt (X-*ComEC*) duplex qPCR assay. The duplex assay achieved optimal specificity and sensitivity when the Xf probes and Xt probes were labelled with FAM and Cy5, a reporter dye combination with clear separation in excitation/emission spectra. Result by one of our TPS participants indicated that different reporter dye pairing might have unintended effects on the probe specificity, optimization on probe reporter chemistry should be considered for future investigations. Our findings showed that this duplex qPCR assay can detect and differentiate both *Xylella* species simultaneously. In comparison, the other published generic qPCR assays could detect both *Xylella* species but lacked the species-differentiation ability (Ito and Suzaki 2017; Ito and Chiaki 2021). The duplex qPCR assay tested in this study is, to the best of our knowledge, the first that can do both. By doing so, this assay improves the efficiency of *Xylella* diagnostics and identification and is particularly useful in a suspected mixed infection.

The assays reviewed in this study were designed for application in regions free of *Xylella* as part of biosecurity measures. Consequently, our evaluation emphasized on the reliable detection of the pathogen's presence/absence, over quantitative performance in terms of qPCR Cq values. The scope of this study was limited by Australian importation restrictions meaning we could not include live *Xylella* cultures, infected-plant or insect materials in our testing panel. Because of that, the analytical sensitivity of assays could not be tested on live spike-in host materials. A new rRNA-based Xt-specific qPCR developed by Su et al. (2023) was published after the commencement of this project and therefore was not included in our evaluation. Further validation studies including more *Xylella* isolates from different hosts and geographic regions, infected plant and vector materials, would be beneficial to advance our understanding of the true potential of both existing and future *Xylella* qPCR assays.

The effectiveness of a diagnostic assay could change when moving from the hands of the assay developers to broad use by end-users in the greater plant pathology community (e.g. diagnosticians, plant pathologists). Complicating factors such as laboratory location, availability of reagents and equipment, and cross-reactivity with local microbiomes could influence the performance of an assay. Taking these factors into careful consideration, this work presented the systematic and collaborative process we undertook in the *Xylella* qPCR assay development and validation. The outcome of this study is incorporated into the Australian NDP (*in final review*) which contains recommended assays for use in Australian plant diagnostic laboratories. In the future, we anticipate that the plant pathology community will benefit from our experience using similar testing frameworks to develop or validate assays for *Xylella* or other emerging plant pathogens.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval Not applicable.

Competing interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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