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All-in-one *Xylella* detection and identification: A nanopore sequencing-compatible conventional PCR

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Abstract

Xylella fastidiosa is a plant-pathogenic bacterium that poses a serious threat to the production of economically important plant species including grapes, almonds, olives and a broad range of amenity plants, causing significant economic losses worldwide. While multiple molecular detection assays have been developed for *X. fastidiosa*, there is a lack of molecular tools available for detection and differentiation of the closely related pear pathogen, *Xylella taiwanensis*. In this study, we present a novel conventional PCR assay with primers that can amplify both *Xylella* species. The amplified product could be sequenced and used for discrimination between the two species and the subspecies within the *fastidiosa* species. This PCR assay was designed using a genome-informed approach to target the *ComEC/Rec2* gene of both *Xylella* species, ensuring a higher specificity than other previously developed PCR assays. A test performance study across five national plant diagnostic laboratories in Australia and New Zealand demonstrated this assay's high sensitivity and specificity to all known species and subspecies within the *Xylella* genus. This PCR assay can be used for *Xylella* identification at the species and subspecies level and is compatible with Sanger sequencing and nanopore sequencing for rapid turnaround time. The newly developed conventional PCR assay presented here offers rapid detection and accurate identification of both *Xylella* species from plant, insect vector or bacterial samples, enabling timely implementation of biosecurity measures or disease management responses.

KEYWORDS
molecular diagnostics, nanopore sequencing, pathogen detection, *Xylella fastidiosa*, *Xylella taiwanensis*

1 | INTRODUCTION

Xylella fastidiosa (Xf) is an emerging plant-pathogenic bacteria associated with severe plant diseases including Pierce's disease in grapevines, citrus variegated chlorosis and almond leaf scorch (Hopkins & Purcell, 2002; Rapicavoli et al., 2018). As of 2022, Xf has been observed to infect a wide and expanding host range, with over 690 plant hosts identified, many of which are agriculturally important such as olive, almond, coffee, mulberry, oleander, peach and lucerne (European Food Safety Authority (EFSA) et al., 2022). Transmitted by sap-feeding

insects or tissue grafting, *Xylella* adheres to and colonizes the walls of xylem vessels, blocking transport of water and nutrients through the plant (Rapicavoli et al., 2018). Symptoms of *Xylella* infection include leaf scorching, leaf browning, wilting of foliage and branches, dieback and death (EPPO, 2019; IPPC, 2018). Currently, there is no treatment once a plant has been infected so preventing the introduction of *Xylella* into new territories is the best control strategy for the pathogen. Taxonomically, Xf is subdivided into three main clades, subspecies *multiplex* (Xfm), subspecies *pauca* (Xfp) and subspecies *fastidiosa*, *morus* and *sandyi* (collectively *fastidiosa*, Xff) based on genomic analysis (Denancé et al., 2019; Marcelletti & Scortichini, 2016). Although Xf was initially described to be the only species within the *Xylella* genus (Wells et al., 1987), a relatively novel *Xylella* lineage that causes pear leaf scorch in Taiwan was identified and described as *Xylella taiwanensis* (Xt; Su et al., 2016). In contrast to Xf, little is known about the distribution outside of Taiwan or pathogenicity of Xt in other plant hosts.

Although *Xylella* poses a serious threat to agricultural industries globally, diagnostics for *Xylella* infection remains difficult. Symptoms of *Xylella* infection vary depending on the host species, stage of infection, the *Xylella* species, subspecies and sequence type (ST), making visual identification of *Xylella*-related diseases a challenging task. Adding to the problem, infected hosts can remain asymptomatic for long periods of time. For instance, infected young olive plants were reported to have a time lag of up to 2 years between initial infection and onset of symptoms (Saponari et al., 2017). Furthermore, *Xylella* is fastidious in nature, slow growing and requires special media for culturing (EPPO, 2019). Due to the broad host range, diverse spectrum of symptoms, long latency period and difficulty in culturing, molecular methods appear to be the most practical options for accurate early detection of *Xylella*-associated disease.

To prevent the spread of *Xylella* into new territories, active research efforts have been put into development of molecular methods for early detection. A range of molecular tests, including conventional PCR (Francis et al., 2006; Ito & Chiaki, 2021; Ito & Suzaki, 2017; Marcolungo et al., 2022; Minsavage et al., 1994), real-time or quantitative PCR (qPCR; Harper et al., 2010; Li et al., 2013; Ouyang et al., 2013) and a loop-mediated isothermal amplification (LAMP) assay (Harper et al., 2010) have been developed and adopted by the plant protection sectors for the detection of Xf (EPPO, 2019; IPPC, 2018). A multilocus sequence typing (MLST) scheme (Yuan et al., 2010) and multiplex qPCR tests (Dupas et al., 2019; Hodgetts et al., 2021) have also been developed to facilitate ST and Xf subspecies determination, respectively. Additionally, nanopore sequencing technology could be used in conjunction with MLST and conventional PCR tests for rapid and sensitive Xf subspecies and ST identification (Faino et al., 2021; Marcolungo et al., 2022). Nanopore sequencing devices such as MinION and Flongle developed by Oxford Nanopore Technologies are portable and have a relatively low start-up cost, making rapid, in-field or near-field deployment of Xf diagnostic tests possible.

While a variety of molecular assays have been developed for specific Xf detection, diagnostic options for Xt remain limited. To date, there is a Xt-specific qPCR assay (Su et al., 2023), a conventional PCR assay and a qPCR assay for *Xylella* generic detection (Ito & Chiaki, 2021; Ito & Suzaki, 2017). Although Xt is deemed to be a lesser threat than Xf, its impact on new hosts and environments is unknown, so effective Xt detection assays are needed for quarantine and management purposes.

Xf has been present in the Americas since the 1800s. It was more recently introduced into parts of Europe. Various Xf outbreaks globally have caused serious impact to olives, grapevines, almonds and many other agricultural industries (Hopkins & Purcell, 2002; Martelli et al., 2016; Schneider et al., 2020). Xf-associated disease management and eradication remains challenging (Strona et al., 2017). Xt is so far reported only in Taiwan, and the associated pear leaf scorch disease is thought to have been present since at least the early 1980s (Leu & Su, 1993). The potential consequences of introducing *Xylella* into new ecosystems, such as Australia, New Zealand and other regions free of *Xylella*, remain uncertain. In this study, we describe the development of a new generic *Xylella* conventional PCR-based assay that is compatible with nanopore sequencing for rapid identification of species and subspecies of *Xylella* for improved diagnostic capability.

2 | MATERIALS AND METHODS

2.1 | Bacterial culture and DNA extraction

A total of 21 isolates of Xf and the type strain of Xt (NCPPB4612; also known as PLS229) from the National Collection of Plant-Pathogenic Bacteria (NCPPB, UK), the Collection for Plant-associated Bacteria (CFBP, France) and the International Collection of Microorganisms from Plants (ICMP, New Zealand) were included in our testing panel (the full list of all bacterial isolates is detailed in Table 1). In addition to *Xylella* species, 24 isolates of *Xanthomonas* and *Stenotrophomonas* from the DAR collection of the New South Wales Plant Pathology & Mycology Herbarium (Orange, Australia) were used to test the specificity of PCR assays.

Xylella isolates were propagated on buffered charcoal yeast extract (BCYE) modified agar at 25°C for 30 days (IPPC, 2018; Wells et al., 1981). The colonies were collected by flooding the agar surface with 0.85% saline solution. The bacterial suspension was then transferred to a microcentrifuge tube and centrifuged at 8000 *g* for 10 min. DNA was extracted from the bacterial pellets using the Blood and Tissue DNeasy kit (Qiagen) according to the manufacturer's instructions and eluted in ultrapure water.

Stenotrophomonas and *Xanthomonas* isolates were cultured on yeast dextrose calcium carbonate (YDC) medium at 25°C for 2 days. A loopful of colonies was collected and mixed with 1 mL of phosphate-buffered saline solution in a microcentrifuge tube. The bacterial suspension was centrifuged at 8000 *g* for 10 min. Similar to the *Xylella* isolates, genomic DNA was extracted from the bacterial pellets using the Blood and Tissue DNeasy kit as per the manufacturer's instructions.

The purity and integrity of the extracted DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), taking absorbance readings at 260 and 280 nm, and by visual observations of DNA bands on 0.8% agarose gels.

The concentration was measured using a Qubit fluorometer (Thermo Fisher Scientific). Based on the Qubit fluorometer reading,

TABLE 1 The specificity testing result of X-*ComEC* PCR comparing to the quantitative PCR (qPCR) described by Harper et al. (2010).

Sample name	Sample origin	Host	Species	Subspecies	X-*ComEC* PCR (this study)	C_t value by Xf qPCR (Harper et al., 2010)
Bacterial culture						
CFBP8071	USA	*Prunus dulcis*	*Xylella fastidiosa*	*fastidiosa*	+	18.54
CFBP8082	USA	*Ambrosia artemisiifolia*	*X. fastidiosa*	*fastidiosa*	+	17.71
ICMP15197	USA	*Vitis vinifera*	*X. fastidiosa*	*fastidiosa*	+	26.64
ICMP8731	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	23.90
ICMP8745	USA	*A. artemisiifolia*	*X. fastidiosa*	*fastidiosa*	+	27.28
NCPPB4432	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	17.65
CFBP7970	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	17.26
ICMP8739	USA	*P. dulcis*	*X. fastidiosa*	*multiplex*	+	20.40
ICMP8740	USA	*Platanus occidentalis*	*X. fastidiosa*	*multiplex*	+	23.47
NCPPB4604	USA	*Vaccinium* sp.	*X. fastidiosa*	*multiplex*	+	18.46
CFBP8173	USA	*Prunus* sp.	*X. fastidiosa*	*multiplex*	+	16.29
ICMP8742	USA	*Ulmus americana*	*X. fastidiosa*	Unknown	+	23.28
CFBP8072	France	*Coffea arabica*	*X. fastidiosa*	*pauca*	+	21.66
CFBP8477	Italy	*Olea europea*	*X. fastidiosa*	*pauca*	+	18.21
CFBP8495	Netherlands	*Coffea arabica*	*X. fastidiosa*	*pauca*	+	18.13
CFBP8073	France	*Coffea canephora*	*X. fastidiosa*	*pauca*	+	18.28
CFBP8524	Italy	*C. arabica*	*X. fastidiosa*	*sandyi*	+	15.64
CFBP8077	USA	*Nerium oleander*	*X. fastidiosa*	*sandyi*	+	17.24
CFBP7969	USA	*Vitis rotundifolia*	*X. fastidiosa*	Unknown	+	19.33
CFBP8073	France	*C. canephora*	*X. fastidiosa*	Unknown	+	17.06
NCPPB4605	USA	*V. vinifera*	*X. fastidiosa*	Unknown	+	18.02
NCPPB4612	Taiwan	*Pyrus pyrifolia*	*Xylella taiwanensis*	Unknown	+	35.63
1622 B strain	Argentina	Unknown	*Xanthomonas fuscans*	*aurantifolii*	–	–
DAR49849	Australia	*Daucus carota*	*Xanthomonas hortorum*	*carotae*	–	–
P03-83	Australia	Unknown	*Xanthomonas alfalfae*	*citrumelo*	–	–
DAR82711	Australia	*Cucurbita moschata*	*Xanthomonas campestris*	*cucurbitae*	–	–
DAR41379	Australia	*Sorghum bicolor*	*Xanthomonas vasicola*	*holcicola*	–	–
DAR82611	Australia	*Oryza* sp.	*Xanthomonas oryzae*	*oryzae*	–	–
DAR82645	Australia	*Phaseolus vulgaris*	*Xanthomonas campestris*	*phaseoli*	–	–
DAR82627	Australia	*Prunus* sp.	*X. campestris*	*pruni*	–	–
DAR35705	Australia	*Triticum aestivum*	*Xanthomonas translucens*	*translucens*	–	–
DAR72015	Australia	*Hordeum vulgare*	*X. translucens*	*translucens*	–	–
DAR30526	Australia	*Lactuca sativa*	*X. campestris*	*vitians*	–	–
DAR65801	Australia	*Citrus paradisi*	*Stenotrophomonas maltophilia*	na	–	–
DAR72045	Australia	*Solanum lycopersicum*	*S. maltophilia*	na	–	–
DAR75512	Australia	Soil	*S. maltophilia*	na	–	–

(Continues)

TABLE 1 (Continued)

Sample name	Sample origin	Host	Species	Subspecies	X-*ComEC* PCR (this study)	C_t value by Xf qPCR (Harper et al., 2010)
DAR76132	Australia	*T. aestivum*	*S. maltophilia*	na	–	–
DAR77232	Australia	Soil	*Stenotrophomonas* sp.	na	–	–
DAR77233	Australia	Soil	*Stenotrophomonas* sp.	na	–	–
DAR77234	Australia	Soil	*Stenotrophomonas* sp.	na	–	–
DAR77236	Australia	Soil	*Stenotrophomonas* sp.	na	–	–
DAR77237	Australia	Soil	*Stenotrophomonas* sp.	na	–	–
DAR33337	Australia	*Prunus salicina*	*Xanthomonas arboricola*	na	–	–
VPRI41552	Australia	*Geranium* sp.	*X. campestris*	na	–	–
DAR82580	Australia	*Jagera pseudorhus*	*Xanthomonas* sp.	na	–	–
DAR73877	Australia	*Capsicum annuum*	*Xanthomonas vesicatoria*	na	–	–
Insect vectors						
Xf-exposed insect-1	USA	*Homalodisca citripennis*	*X. fastidiosa*	*multiplex*	+	26.85
Xf-exposed insect-2	USA	*H. citripennis*	*X. fastidiosa*	*multiplex*	+	30.70
Xf-exposed insect-3	USA	*H. citripennis*	*X. fastidiosa*	*multiplex*	+	32.75
Xf-exposed insect-4	USA	*H. citripennis*	*X. fastidiosa*	*multiplex*	+	28.59
Xf-exposed insect-5	USA	*H. citripennis*	*X. fastidiosa*	*multiplex*	+	32.57
Xf-exposed insect-6	USA	*H. citripennis*	*X. fastidiosa*	*multiplex*	+	35.53
Plant samples						
Healthy *Vitis* S4	USA	*V. vinifera*	Not infected	Not applicable	–	–
Vitis Xf DNA1 NGS	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	27.11
Vitis Xf DNA2 NGS	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	27.02
Xf cocit plants B1S1	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	–	33.76
Xf cocit plants B1S2	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	26.22
Xf cocit plants B1S3	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	26.03
Xf cocit plants B1S4	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	27.82
Xf cocit plants B2S1	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	26.50
Xf cocit plants B2S2	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	25.95
Xf cocit plants B2S3	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	28.18

TABLE 1 (Continued)

Sample name	Sample origin	Host	Species	Subspecies	X-*ComEC* PCR (this study)	C_t value by Xf qPCR (Harper et al., 2010)
Xf cocit plants B2S4	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	27.73
Xf cocit plants B2S5	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	25.30
Xf cocit plants B2S6	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	24.39
Xf cocit plants B2S7	USA	*V. vinifera*	*X. fastidiosa*	*fastidiosa*	+	33.27
Xf cocit plants B3S1	USA	*Olea europea*	*X. fastidiosa*	*fastidiosa*	+	26.48
Xf cocit plants B3S2	USA	*O. europea*	*X. fastidiosa*	*fastidiosa*	+	31.86
Xf cocit plants B4S1	USA	*O. europea*	*X. fastidiosa*	*fastidiosa*	+	27.35
Xf cocit plants B4S2	USA	*O. europea*	*X. fastidiosa*	*fastidiosa*	+	26.88
Xf cocit plants B5S1	USA	*N. oleander*	*X. fastidiosa*	*fastidiosa*	+	23.98

Abbreviation: na, not applicable.

a series of 10-fold serial dilutions of bacterial culture DNA samples was prepared from 0.24 ng/μL to 0.24×10^{-10} ng/μL to determine the limit of detection. The number of DNA copies per μL was estimated with the approximate *Xylella* genome size at 2,540,000 bp using the following formula:

$$\text{Number of copies} = \frac{\text{DNA quantity (ng)} \times \text{Avogadro's constant}}{\text{Length (bp)} \times \text{Conversion factor} \times \text{Average mass of 1 bp dsDNA}}$$

where average mass of 1 bp double-stranded (ds) DNA = 660 g/mol, Avogadro's constant = 6.022×10^{23}, conversion factor = 10^9.

2.2 | Plant and insect samples and DNA extraction

Six insect samples and 19 plant samples were included in the testing panel (Table 1). These samples were provided by a collaborator and were originally sourced from the Plant Pest Diagnostic Centre, California and University of California (Berkley), California, United States. Xfm-infected *Homalodisca vitripennis* (laboratory-reared by the Agricultural Research Service, United State Department of Agriculture) preserved in alcohol were dried using paper towels and ground in a microcentrifuge tube using a micropestle before DNA extraction using the Blood and Tissue DNeasy kit as previously mentioned.

Naturally infected plant samples (petioles for grapevine and midrib for all other plants) sourced from the United States were harvested and ground to a fine powder in liquid nitrogen with a mortar and pestle. A total of 100 mg of ground tissue was then resuspended in AP1 lysis buffer and DNA extracted using a DNeasy Plant Mini Kit (Qiagen). DNA extraction was performed as per the manufacturer's instruction, with the amendment of four wash steps instead of two at steps 9 and 10. Quality and concentration of extracted DNA were assessed as described above.

2.3 | Primer design

Primer pairs specific to the *Xylella* genera were designed using RUCS (Thomsen et al., 2017). Briefly, a collection of 'positive' genomes including 88 strains of *Xylella* (86 Xf strains and 2 Xt strains) and a collection of 'negative' genomes including 49 strains of *Stenotrophomonas* and 210 strains of *Xanthomonas* were downloaded from the NCBI RefSeq or GenBank database (Table S1). The RUCS tool compared the sequences of the 'positive' and 'negative' collections to find unique sequences only present in *Xylella* genomes and identify PCR primer pairs for these unique sequences. Primer pairs that amplified only in the coding sequence (CDS) region and targeted a single region were filtered using in-house scripts (https://github.com/bogemad/rucs_analysis). Preliminary screening (data not shown) identified a primer pair (X-*ComEC*-F/R) that targets the *comEC/Rec2* gene in *Xylella* spp., which is the focus of this study.

2.4 | In silico PCR amplicon analysis

A collection of 120 *Xylella* genomes were downloaded from the NCBI RefSeq or GenBank database (Table S2). To identify the

expected amplicon sequence from each of the *Xylella* genomes, an open-sourced in silico PCR script (https://github.com/egonozer/in_silico_pcr) was used with setting -m 2 (allow up to 2 mimatches per primer sequence) and -l 0 (no indels allowed). The identified amplicon sequences were then imported into the sequence analysis software Geneious Prime (v. 2021.2.2) and aligned using MAFFT alignment algorithm (Katoh et al., 2002). The resultant alignment was used for building a midpoint-rooted maximum-likelihood phylogenetic tree using IQ-TREE2 with setting of 1000 ultrafast bootstrap iterations (-B 1000) with best-fit model selection by ModelFinder Plus (-m MFP) (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). The resultant phylogenetic trees were then exported and visualized using the interactive Tree of Life (v. 6.5.2; Letunic & Bork, 2019). The sequences of X-*ComEC* primers designed in this study and the primer sets described by Minsavage et al. (1994), Ito and Chiaki (2021) and Marcolungo et al. (2022) were compared to the aligned genomes using the aforementioned in silico PCR script to determine the potential to amplify known *Xylella* spp., subspecies and ST. For the primer sets described by Minsavage et al. (1994) and Marcolungo et al. (2022), additional analyses were performed to find potential binding sites in the Xt genome. The primer sets were queried for binding sites in the Xt RefSeq representative genome (NCPPB4612) using the Primer-BLAST tool with default setting (Ye et al., 2012). A BLASTn query was also performed using the Xf target sequences of these two sets of primers to search for sequence similarity in the Xt genome. The results of this in silico analysis were confirmed by wet laboratory analysis in which representative isolates of the four *Xylella* lineages, including CFBP4612 (Xt), ICMP8731 (Xff), ICMP8739 (Xfm) and CFBP8072 (Xfp), were tested using each of the four PCR assays, followed by Sanger sequencing of the amplified product.

2.5 | PCR amplification

In total, four PCR primer sets were compared across representative isolates of the four *Xylella* lineages, including the primer sets described by Minsavage et al. (1994), Ito and Chiaki (2021), Marcolungo et al. (2022) and the X-*ComEC* primer set presented in this study (Table 2). For each reaction, a 20 μL reaction mix was prepared with 10 μL of MyTaq HS 2× reaction mix (Meridian Bioscience), 500 nM forward primer, 500 nM reverse primer and 2 μL of DNA templates. PCRs were performed in triplicate on a thermocycler with reaction conditions as listed in Table S3. Amplification was confirmed by gel electrophoresis in a 1% agarose gel for 30 min at 100 V. For positive samples, the amplicons were purified using ISOLATE II PCR and Gel kit (Meridian Bioscience) and sent for Sanger sequencing at the Australian Genome Research Facility Ltd.

Using the same reaction mix and conditions, further testing of the X-*ComEC*-F/R primer set was performed in triplicate using test samples listed in Table 1. To confirm the presence of Xf in the test samples, the Xf qPCR assay described by Harper et al. (2010) was performed on a QuantStudio 5 real-time PCR machine (Applied Biosystems). The 10 μL reaction mix contained 0.1 μL Immolase DNA polymerase (Meridian Bioscience), 1 μL of 10× Immobuffer, 1 mM dNTP, 6 mM $MgCl_2$, 300 nM Xf-R primer, 300 nM Xf-F primer, 200 nM Xf-P probe and 1 μL of DNA template. The primer sequences and reaction conditions were the same as those detailed in the original paper of Harper et al. (2010). Samples with a cycle threshold (C_t) value of <38 cycles were considered a positive amplification.

2.6 | Interlaboratory test performance study

After the X-*ComEC* PCR protocol had been established, biologists from five national plant diagnostic laboratories across Australia and New Zealand participated in an interlaboratory test performance study (TPS) as per EPPO (PM 7/122 (2), 2014). This was to evaluate the robustness of this PCR method when different PCR reagents and equipment were used in different laboratory settings (Table S4). During the TPS, the X-*ComEC* PCR mix was prepared using the same concentration of primers and volume of DNA templates with polymerases listed in Table S1 and their associated proprietary reaction mixes. The PCR was performed with reaction conditions and cycle numbers as mentioned previously unless stated otherwise. Samples were processed in triplicate.

The TPS consisted of three testing exercises:

1. Specificity exercise. A testing panel consisted of DNA extracted from representative isolates of four different *Xylella* linages including Xff (ICMP8731), Xfm (ICMP8739), Xfp (CFBP8072)

TABLE 2 Primer sets compared for *Xylella* spp. detection in this study and their sequences.

Primer	Sequence (5′–3′)	Amplicon length (bp)	References
HL5 (forward)	AAGGCAATAAACGCGCACTA	900	Marcolungo et al. (2022)
HL-ONT (reverse)	AAGCGCTTTACCGACTCAAA		
RTS31 (forward)	GCGTTAATTTTCGAAGTGATTCGATTGC	733	Minsavage et al. (1994)
RTS33 (reverse)	CACCATTCGTATCCCGGTG		
X67S1 (forward)	GGACGGCAGCACATTGGTA	604	Ito and Chiaki (2021)
XL2r (reverse)	CCTCTACCACACTCTAGCTATC		
X-ComEC-F (forward)	AGTCATGCTGATAGTGATCACGT	650	This study
X-ComEC-R (reverse)	CAGCATGTCTCGTTTCTCCGA		

TABLE 3 Comparison of nanopore sequencing results between the three consensus calling methods of the nanopore sample test.

					De novo sequence cluster method			Reference-guided method			Geneious method		
Sample ID	True identity	Read count	N50	Mapped reads (%)	Result	Nucleotide identity (%)	Query coverage (%)	Result	Nucleotide identity (%)	Query coverage (%)	Result	Nucleotide identity (%)	Query coverage (%)
Bacterial culture													
ICMP8731	Xff	36,345	412	99.63	+	98.92	85.54	+	100	99.38	+	100	96.62
ICMP8739	Xfm	24,106	417	99.78	+	100	96.15	+	100	99.38	+	100	97.38
ICMP8740	Xfm	24,316	409	99.19	+	99.18	93.69	+	100	98.77	+	100	96.62
ICMP8745	Xff	24,622	414	99.82	+	99.16	91.69	+	100	99.38	+	100	97.54
NCPPB4432	Xff	33,901	412	99.77	+	99.02	93.85	+	100	100	+	100	96.92
CFBP8495	Xfp	48,794	414	99.82	+	98.45	84.46	+	100	99.08	+	100	96.92
CFBP8072	Xfp	37,609	412	99.73	+	99.66	91.54	+	100	99.85	+	100	96.62
CFBP8077	Xff	23,183	413	99.77	+	100	95.08	+	100	99.38	+	100	96.92
NCPPB4612	Xt	36,998	423	97.51	+	98.56	96.00	+	100	99.85	+	100	96.62
Plant samples and insect vectors													
Xf *Vitis* stock	Xff	91	120	18.05	nd	na	na	Xff[a]	97.55	94.15	nd	na	na
Xf plant B2S4	Xff	21,572	416	99.73	+	99.52	96.77	+	100	99.54	+	100	96.92
Xf plant B3S1	Xff	14,780	417	98.96	+	99.84	95.23	+	100	99.85	+	100	96.92
Xf plant B4S2	Xff	10,905	423	99.54	+	99.83	90.31	+	100	99.38	+	100	97.08
Xf plant B5S1	Xff	28,187	418	99.67	+	98.83	92.31	+	100	99.08	+	100	96.62
Xf-exposed insect-1	Xfm	17,612	424	98.16	+	99.53	97.08	+	100	99.23	+	100	96.92

Abbreviations: +, correct and valid identification; na, not applicable; nd, not detected; Xff, *Xylella fastidiosa* subsp. *fastidiosa*; Xfm, *X. fastidiosa* subsp. *multiplex*; Xfp, *X. fastidiosa* subsp. *pauca*; Xt, *Xylella taiwanensis*.

[a]Indeterminate result.

TABLE 4 Comparison of nanopore sequencing results from pure culture DNA samples between the three consensus calling methods of the nanopore sensitivity test.

							De novo sequence cluster method		
Sample	Concentration (copies/µL)	Concentration (ng/µL)	PCR band	Read count	N50	Mapped reads (%)	Result	Nucleotide identity (%)	Query coverage (%)
ICMP 8731 Xff	87,540	2.40e−01	+	92,041	502	99.64	+	97.51	86.62
	8754	2.40e−02	+	62,406	496	99.69	+	98.46	89.85
	875.4	2.40e−03	+	75,727	509	99.71	+	99.49	90.00
	87.54	2.40e−04	+	112,689	501	99.66	+	91.71	83.54
	8.754	2.40e−05	+	49,558	507	99.41	+	98.65	91.38
	0.8754	2.40e−06	+	44,893	505	99.45	+	97.97	91.08
	0.08754	2.40e−07	−	3406	237	2.64	Xff[a]	99.55	34.31
	0.008754	2.40e−08	−	2321	235	6.49	nd	na	na
	0.0008754	2.40e−09	−	6126	239	4.63	nd	na	na
	0.00008754	2.40e−10	−	1997	237	6.63	nd	na	na
ICMP 8739 Xfm	87,540	2.40e−01	+	54,235	507	99.70	+	95.43	87.54
	8754	2.40e−02	+	36,711	503	98.85	+	99.11	86.00
	875.4	2.40e−03	+	53,018	506	99.22	+	100	87.85
	87.54	2.40e−04	+	57,127	503	99.19	+	98.36	93.54
	8.754	2.40e−05	+	36,415	508	98.86	+	98.30	90.62
	0.8754	2.40e−06	+	47,219	503	99.53	+	99.31	88.62
	0.08754	2.40e−07	−	1571	247	1.57	Xfp[b]	98.50	51.23
	0.008754	2.40e−08	−	863	232	4.15	Xfm[a]	98.31	9.08
	0.0008754	2.40e−09	−	2163	233	1.37	Xfm[a]	95.51	13.69
	0.00008754	2.40e−10	−	1332	243	5.80	nd	na	na
CFBP8072 Xfp	87,540	2.40e−01	+	63,667	503	99.67	+	98.79	76.31
	8754	2.40e−02	+	60,526	512	99.66	+	99.66	90.15
	875.4	2.40e−03	+	40,141	507	99.41	+	98.55	84.77
	87.54	2.40e−04	+	37,565	487	98.03	+	91.37	69.54
	8.754	2.40e−05	+	26,780	502	99.09	+	99.27	84.62
	0.8754	2.40e−06	+	6791	550	97.48	Xff[c]	95.68	92.62
	0.08754	2.40e−07	−	893	241	3.49	Xfp[a]	99.12	17.38
	0.008754	2.40e−08	−	1799	233	2.34	Xfm[b]	100	9.54
	0.0008754	2.40e−09	−	2771	353	82.04	Xfp[a]	100	84.46
	0.00008754	2.40e−10	−	1378	244	26.50	Xfp[a]	99.11	34.46
NCPPB 4612 Xt	87,540	2.40e−01	+	42,468	502	96.96	+	99.63	83.85
	8754	2.40e−02	+	44,333	517	97.12	+	100	91.38
	875.4	2.40e−03	+	50,951	498	94.56	+	99.27	84.62
	87.54	2.40e−04	+	35,173	502	97.82	+	99.30	87.54
	8.754	2.40e−05	+	34,747	504	98.30	+	98.91	84.31
	0.8754	2.40e−06	−	869	225	4.78	nd	na	na
	0.08754	2.40e−07	−	3476	234	1.90	nd	na	na
	0.008754	2.40e−08	−	1889	243	1.98	Xt[a]	99.06	16.31
	0.0008754	2.40e−09	−	3067	239	1.74	nd	na	na
	0.00008754	2.40e−10	−	1063	334	5.48	nd	na	na
No-template control 1 (NTC1)			−	864	−	0.69	nd	na	na
No-template control 2 (NTC2)			−	538	−	2.19	nd	na	na

Abbreviations: +, correct and valid identification; na, not applicable; nd, not detected; Xff, *Xylella fastidiosa* subsp. *fastidiosa*; Xfm, *X. fastidiosa* subsp. *multiplex*; Xfp, *X. fastidiosa* subsp. *pauca*; Xt, *Xylella taiwanensis*.

[a]Indeterminate result.

[b]Indeterminate result and misidentification.

[c]Misidentification.

Reference-guided method			Geneious method		
Result	Nucleotide identity (%)	Query coverage (%)	Result	Nucleotide identity (%)	Query coverage (%)
+	100	100	Xfp/Xff	96.63	73.08
+	100	100	Xfp/Xff[a]	93.85	67.54
+	100	100	Xfp/Xff[a]	79.50	55.54
+	100	99.38	Xfp/Xff[a]	93.95	63.54
+	100	99.23	Xff[a]	87.70	66.31
+	100	99.23	+	92.78	72.46
Xff[a]	100	97.08	nd	na	na
Xff/Xfm[a]	99.84	97.08	nd	na	na
Xff[b]	100	97.08	nd	na	na
Xfp[b]	100	97.08	nd	na	na
+	100	100	Xfm[a]	93.18	65.38
+	100	100	+	93.85	80.00
+	100	100	Xfm[a]	91.91	68.46
+	100	100	Xfm[a]	91.23	64.92
+	100	99.85	+	95.51	75.38
+	100	99.08	Xfm[a]	91.02	65.08
Xff[b]	99.05	96.62	nd	na	na
Xff[b]	96.84	97.23	nd	na	na
Xff[b]	100	68.31	nd	na	na
Xff[b]	100	97.08	nd	na	na
+	100	100	Xfp/Xff[a]	92.34	66.31
+	100	100	Xfp/Xff	97.50	73.69
+	100	100	Xfp/Xff[a]	92.96	65.54
+	100	100	+	98.57	75.08
+	100	99.54	Xfp/Xff[a]	90.99	66.62
+	99.85	99.54	+	98.7	76.31
Xfp[a]	98.72	96.46	nd	na	na
Xfm[b]	99.53	97.85	nd	na	na
Xfp[a]	100	97.54	nd	na	na
Xfp[a]	100	97.23	nd	na	na
+	100	100	+	98.34	74.15
+	100	100	+	96.34	71.38
+	100	100	Xt[a]	97.99	68.77
+	100	99.23	+	91.60	73.23
+	100	99.54	Xt[a]	95.901	67.69
Xfp[b]	96.98	96.92	nd	na	na
Xff[b]	100	97.08	nd	na	na
Xfp[b]	99.05	96.62	nd	na	na
Xff[b]	99.36	96.77	nd	na	na
Xff[b]	100	97.23	nd	na	na
Xfm[b]	95.69	78.46	nd	na	na
Xfm[b]	99.03	94.92	nd	na	na

	RST31/RST33 (Minsavage et al., 1994)	HL5/HL-ONT (Marcolungo et al., 2022)	X67S1/XL2r (Ito & Chiaki, 2021)	X-*ComEC*-F/R (this study)
Xff	No mismatch	**Target (CFBP8073)** 3'-TTCCGTTATTTGTGCGTGAT-5' 5'-AAGGCAATAAACGCGCACTA-3' **Forward primer (HL5)** **Target (CFBP8073)** 3'-TTTGAGTCGGTAGAGCGCTT-5' 5'-AAACTCAGCCATTTCGCGAA-3' **Reverse primer (HL-ONT)**	No mismatch	No mismatch
Xfm	No mismatch	No mismatch	No mismatch	No mismatch
Xfp	**Target (Salento-1)** 3'-CGCAATTAAAAGCTTCACTAAGCTAAAG-5' 5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3' **Forward primer (RTS31)**	No mismatch	**Target (6c)** 3'-CCTGCCGTCGTGCAACCAT-5' 5'-GGACGGCAGCACATTGGTA-3' **Forward primer (X67S1)**	No mismatch
Xt	No binding	No binding	**Target (PLS229)** 3'-CTTGCCGTCGTGTCACCAT-5' 5'-GGACGGCAGCACATTGGTA-3' **Forward primer (X67S1)**	No mismatch

FIGURE 1 Comparison of primer specificity of three published primer sets and the X-*ComEC* primer set towards genomes of *Xylella taiwanensis* (Xt) and three different *Xylella fastidiosa* subspecies, *fastidiosa* (Xff), *multiplex* (Xfm) and *pauca* (Xfp). A total of 120 publicly available *Xylella* genomes were examined for their binding with these primer sets by in silico PCR. Examples of primer–target mismatch and their locations are shown in red. No mismatch = the primer set aligned to all genomes with no mismatches. No binding = the primer set could not bind to any genomes within the *Xylella* lineage.

	Xff-amp1	Xff-amp2	Xff-amp3	Xfm-amp1	Xfm-amp2	Xfm-amp3	Xfp-amp1	Xfp-amp2	Xfp-amp3	Xfp-amp4	Xt-amp1
Xff-amp1		1	2	4	3	4	14	4	5	2	76
Xff-amp2	1		1	3	2	3	13	3	4	1	75
Xff-amp3	2	1		4	3	4	14	4	5	2	76
Xfm-amp1	4	3	4		1	2	16	6	7	4	78
Xfm-amp2	3	2	3	1		1	15	5	6	3	77
Xfm-amp3	4	3	4	2	1		16	6	7	4	78
Xfp-amp1	14	13	14	16	15	16		10	11	12	75
Xfp-amp2	4	3	4	6	5	6	10		1	2	74
Xfp-amp3	5	4	5	7	6	7	11	1		3	75
Xfp-amp4	2	1	2	4	3	4	12	2	3		74
Xt-amp1	76	75	76	78	77	78	75	74	75	74	

FIGURE 2 The matrix table shows the number of single-nucleotide polymorphisms (SNPs) between all known X-*ComEC* PCR amplicon sequence variants (amp1 to amp4) derived from *Xylella taiwanensis* (Xt) and three different *X. fastidiosa* subspecies, *fastidiosa* (Xff), *multiplex* (Xfm) and *pauca* (Xfp). The green-to-yellow gradient indicates the number of SNPs from high to low.

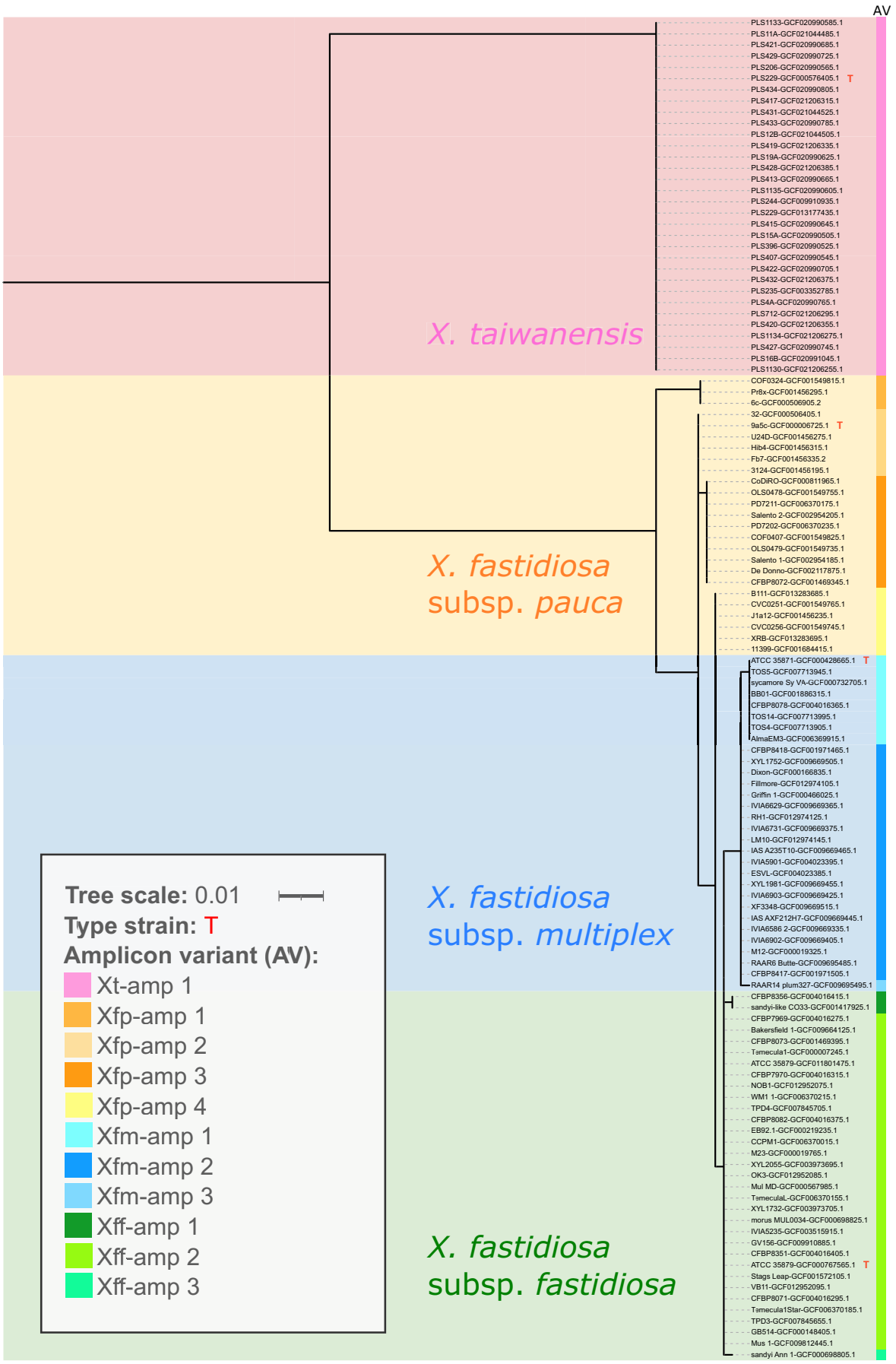
and Xt (NCPPB4612), and nontarget isolates (15 *Xanthomonas* and nine *Stenotrophomonas* as described in Table 1). All were provided to each laboratory. Participants reported amplification from these samples using the X-*ComEC* PCR assay.

2. Cross-reactivity exercise. Participants were instructed to perform the X-*ComEC* PCR assay on uninfected plant hosts to assess presence/absence of off-target amplification with the DNA of these plants and their associated microbes. Collectively, 161 uninfected plant samples representing 23 *Xylella* host genera across different locations in Australia, New Zealand and Europe were collected; midribs were processed from collected hosts with the exception of grape, where the petioles were used. DNA was extracted using the Qiagen Plant and Tissue Kit, as per the manufacturer's instructions, and tested with the X-*ComEC* PCR assay (Table S5). These samples included commercially important hosts of *Xylella* including *Citrus*, *Vitis*, *Olea* and *Prunus* species.
3. Sensitivity exercise. A set of DNA samples from each representative isolate of the four different *Xylella* linages as previously mentioned were diluted to 2.4 ng/μL and distributed to all participating laboratories. Using each DNA sample, each participant prepared a dilution series of 10 different concentrations from 0.24 ng/μL to 2.4×10^{-10} ng/μL and tested the detection limit of X-*ComEC* PCR.

2.7 | Nanopore sequencing

To validate this PCR method as a rapid diagnostic tool, the amplicons from the *Xylella*-positive samples (including bacterial culture, plants and insects) were sequenced using nanopore sequencing devices.

FIGURE 3 Comparison of the X-*ComEC* PCR amplicon variants (AV) of 120 *Xylella* genomes corresponding to *Xylella taiwanensis* (Xt) and *X. fastidiosa* subspecies *pauca* (Xfp), *fastidiosa* (Xff) and *multiplex* (Xfm). The midpoint-rooted maximum-likelihood phylogenetic is inferred by using IQtree2 with best model selected by ModelFinder Plus algorithm and 1000 iterations of ultrafast bootstrapping tests.



Two sequencing testing runs (nanopore sample test and nanopore sensitivity test) were performed. The nanopore sample test aimed to evaluate the performance of nanopore PCR amplicon sequencing in differentiating between different *Xylella* lineages. In the nanopore sample test, the testing DNA extracts were derived from nine bacterial culture samples, five naturally infected plant samples and an Xf insect vector sample (Table 3). The nanopore sensitivity test run evaluated the detection limit of nanopore sequencing on PCR amplicons produced by bacterial culture DNA templates in different concentrations (Table 4). PCR amplicons generated by a ring testing participant using 40 cycles during the sensitivity testing exercise were used as inputs. These included no-template control (NTC) samples, positive-band (PB) samples with detectable bands and no-band (NB) samples with amplification products that were undetectable in gel electrophoresis.

For both runs, the PCR products were purified by AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. Purified PCR products were then quantified using a NanoDrop spectrophotometer and a Qubit fluorometer (Thermo Fisher Scientific). Of each purified PCR product, 50 ng was used for library preparation using the Rapid sequencing gDNA-barcoding kit (Oxford Nanopore Technologies). When the total DNA quantity was less than 50 ng, all of the PCR product was used in library preparation. The preparation was done following the manufacturer's instructions, employing the short fragment buffer (SFB) for the wash steps. The sequencing library was loaded onto the MinION flow cell (Oxford Nanopore Technologies, v. R9.4.1), and sequencing was performed using the MinKNOW software (Oxford Nanopore Technologies, v. 22.12.5).

2.8 | Sequencing data analysis

Basecalling was performed with Guppy basecaller (v. 6.1.5) with high accuracy configuration file (dna_r9.4.1_450bps_hac.cfg) and a minimum quality score filter >7 (-min_qscore 7). Demultiplexing and adapter-trimming were performed with the Guppy barcoder (v. 6.1.5). Sequencing quality check was performed using the 'faster' tool (https://github.com/angelovangel/faster). Reads were mapped against all known X-*ComEC* PCR amplicon sequence variants using Minimap2 (v. 2.17-r941; Li, 2021). Using the resulting fastq files as input, three different approaches were tested to generate consensus sequences:

1. De novo sequence cluster method. The NGSpeciesID tool (v. 0.1.3; Sahlin et al., 2021) was used to generate de novo consensus sequences from the fastq file. This method does not require entering a reference sequence.
2. Reference-guided method. The consensus sequence was generated using medaka (v. 0.11.5) with the 'medaka_consensus' command (https://github.com/nanoporetech/medaka). This method requires the input of a reference sequence, and in this case, the Xff isolate ICMP8731 amplicon sequence was used as the reference.
3. Geneious method. The consensus sequence was generated using Geneious Prime (Dotmatrics; v. 2021.2.2). Raw reads were first sorted from longest to shortest; then reads within the 650 ± 100 bp in length were randomly sampled. When the total read counts were more than 1000, approximately 1000 reads were randomly sampled and used for the analysis. Using MAFFT alignment, these sequences were aligned and then used to generate consensus sequences with base matching threshold set as 50%.

Finally, to assess the species/subspecies identification accuracy, we searched these consensus sequences against all X-*ComEC* PCR amplicon sequence variants using BLASTn (v. 2.9.0). Only the closest match with the highest nucleotide identity and query coverage was reported as the identification result. The scripts for running method 1 and method 2 are detailed in the github page https://github.com/chewbeckie/Xylella_pcrONT.

2.9 | Sequencing results interpretation

Four criteria were put into consideration: (a) a positive amplification at 650 bp should be detectable using gel electrophoresis; (b) N50 value (shortest contig length to cover 50% of the genome) ≥200 bp; (c) at least 50% of all reads should map to any X-*ComEC* PCR amplicon sequence variant; and (d) BLASTn search result should have a nucleotide identity percentage ≥90% and a query coverage ≥70%.

A valid *Xylella* species/subspecies identification should meet all of the above criteria. If the BLASTn search produced a match to X-*ComEC* PCR amplicons but the sample did not meet all four criteria, it was categorized as an indeterminate result. If the BLASTn search did not produce any matches to the X-*ComEC* PCR amplicon sequences, it was concluded that *Xylella* was not detected in these samples.

3 | RESULTS

3.1 | X-*ComEC* PCR primer homology

Using genome-informed diagnostic development, a primer pair X-ComEC-F and X-ComEC-R was designed that amplifies the region towards the 5′ end of the *comEC/Rec2* gene encoding a DNA internalization-related competence protein comEC/Rec2 (Pimentel & Zhang, 2018).

In silico PCR analysis showed that the X-*ComEC* primer pair could generate a single 650 bp PCR product from each of the total 120 *Xylella* genomes analysed (Table S2). The same in silico PCR analysis and a Primer-BLAST search demonstrated that the RST31/RST33 (Minsavage et al., 1994) and HL5/HL-ONT (Marcolungo et al., 2022) primer sets were unlikely to be able to amplify Xt (Figure 1). Sequence comparison showed that the targeted loci of these two primer sets in the Xt genome was too diverse from that of Xf; therefore, no binding sites could be identified. BLASTn search of a RST31/RST33 amplicon sequence on the RefSeq Xt

(a)

Sample	Concentration (copies/µl)	Concentration (ng/µl)	Participant	A	B	B	C	D	E	F	F
			PCR cycle	35	35	40	35	35	35	35	40
			Xt qPCR Ct value	Result							
ICMP 8731 *Xff*	87540	2.40E-01	22.84								
	8754	2.40E-02	26.40								
	875.4	2.40E-03	30.29								
	87.54	2.40E-04	33.56								
	8.754	2.40E-05	35.79								
	0.8754	2.40E-06	nd								
	0.08754	2.40E-07	nd								
	0.008754	2.40E-08	nd								
	0.0008754	2.40E-09	nd								
	0.00008754	2.40E-10	nd								
ICMP 8739 *Xfm*	87540	2.40E-01	21.70								
	8754	2.40E-02	26.21								
	875.4	2.40E-03	29.55								
	87.54	2.40E-04	32.96								
	8.754	2.40E-05	35.12								
	0.8754	2.40E-06	nd								
	0.08754	2.40E-07	nd								
	0.008754	2.40E-08	nd								
	0.0008754	2.40E-09	nd								
	0.00008754	2.40E-10	nd								
CFBP8072 *Xfp*	87540	2.40E-01	19.53								
	8754	2.40E-02	24.17								
	875.4	2.40E-03	33.86								
	87.54	2.40E-04	35.91								
	8.754	2.40E-05	nd								
	0.8754	2.40E-06	nd								
	0.08754	2.40E-07	nd								
	0.008754	2.40E-08	nd								
	0.0008754	2.40E-09	nd								
	0.00008754	2.40E-10	nd								
NCPPB 4612 *Xt*	87540	2.40E-01	nd								
	8754	2.40E-02	nd								
	875.4	2.40E-03	nd								
	87.54	2.40E-04	nd								
	8.754	2.40E-05	nd								
	0.8754	2.40E-06	nd								
	0.08754	2.40E-07	nd								
	0.008754	2.40E-08	nd								
	0.0008754	2.40E-09	nd								
	0.00008754	2.40E-10	nd								

(b)

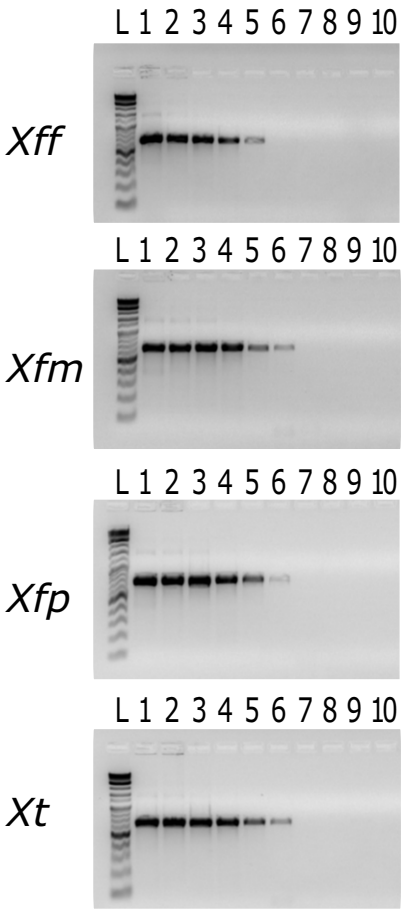


FIGURE 4 The comparison of X-*ComEC* PCR performance based on the interlaboratory test performance study performed by participants from five national plant diagnostic laboratories across Australia and New Zealand. (a) Results of X-*ComEC* PCR sensitivity testing exercise in comparison with the cycle threshold (C_t) value generated by Xf quantitative PCR (qPCR) assay described by Harper et al. (2010). No detection qPCR results are denoted as nd. Four *Xylella* isolates represent different lineages used 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 colour indicates presence of strong bands (blue), faint bands (yellow) or absence of bands (red) based on the gel electrophoresis results of X-*ComEC* PCR. (b) The gel electrophoresis result of a ring testing participant using 40 PCR cycles.

representative genome revealed that only the first 507 bp out of the 734 bp amplicon sequence were matching between Xt and Xf. Similarly, BLASTn query of a HL5/HL-ONT amplicon sequence on the RefSeq Xt representative genome showed that only the 41st to 831st bp of the 904 bp amplicon sequence were matching to the Xt genome.

While both the X67S1/XL2r (Ito & Chiaki, 2021) and the X-*ComEC* primer sets were predicted to amplify Xt, the latter was predicted to have greater homology as there were no mismatches in the primer binding sites in the genome sequences of Xt, whereas two mismatches were found in the forward primer binding site of the X67S1/XL2r primer set (Figure 1). The X67S1 forward primer also had one mismatch to nine Xfp genomes. Similarly, mismatches to Xff genomes were found amongst the other primer sets in previous studies. One or two mismatches were found in the HL5/HL-ONT binding site on 32 Xff genomes (Figure 1). A mismatch was also identified in the RST31/RST33 primer set on nine of the Xfp genomes (Figure 1). In contrast to these primer sets, the X-*ComEC* primer set had no observed mismatches in any *Xylella* genome. Preliminary wet laboratory analysis using representative isolates from the four *Xylella* lineages confirmed the in silico prediction that only X67S1/XL2r and X-ComEC primer sets could correctly detect Xt and all Xf subspecies (data not shown). It is of note that the effect of these mismatches we identified in these other primer sets is unknown for the actual PCR efficiency to various Xf and Xt isolates. Further wet laboratory experimentation would be required for confirmation.

3.2 | X-*ComEC* PCR detection of *Xylella* from bacterial, plant and insect samples

The specificity of the X-*ComEC* primer set was tested comprehensively across a DNA testing panel consisting of 46 bacterial monoculture samples (Table 1). The X-*ComEC* primer set successfully amplified all *Xylella* bacterial monoculture samples in our testing panel, including 21 isolates of Xf and one isolate of Xt (NCPPB4612). In addition, the X-*ComEC* PCR did not produce any false-positive amplifications using DNA from the closely related, nontarget isolates included in the panel. The TPS conducted by all five independent laboratories confirmed the specificity of the *X-ComEC* PCR; amplicons were generated from DNA of the four representative *Xylella* isolates in the testing panel but not from the DNA of the 24 nontarget isolates (data not shown). This demonstrated that the *X-ComEC* PCR can perform consistently, while maintaining specificity to *Xylella* when different PCR reagents and equipment were used.

In addition to DNA extracted from bacterial cultures, the X-*ComEC* PCR successfully detected *Xylella* in DNA extracted directly from infected plants and insects (Table 1). The presence of Xf in these samples was confirmed by PCR amplification using the qPCR of Harper et al. (2010) (Table 1). Additionally, the cross-reactivity exercise of the TPS demonstrated that the X-*ComEC* PCR did not produce off-target amplification when the 161 locally (Australia and New Zealand) collected plant materials were tested (Table S5).

3.3 | X-*ComEC* PCR differentiation of *Xylella* at species and subspecies level

The X-*ComEC* PCR amplicon sequence regions were extracted from a collection of 120 *Xylella* genomes and compared with each other to evaluate the sequence divergence of this region and its usefulness as a diagnostic marker. Altogether, up to four different amplicon sequence variants were identified in each Xf subspecies, whereas only one amplicon variant was found amongst the Xt genomes (Figure S1; Data S1). We compared the single-nucleotide polymorphism (SNP)-sites that correlated with the specific species and subspecies of *Xylella*. Intraspecific comparison between Xf subspecies revealed up to 16 SNPs amongst different amplicon variants (Figure 2). Conserved SNPs within all amplicon variants of a Xf subspecies could be identified (Figure S1). For instance, a G-to-A substitution was found at the 90th nucleotide position (5′ to 3′ direction) of all three Xfm amplicon variants. At the 472nd nucleotide position (5′ to 3′ direction) of all four Xfp amplicon variants, C was replaced by G. Interspecific comparison showed that Xt isolates had the most distinctive amplicon sequence from Xfm, with 77 to 78 SNPs identified between the two lineages (Figure 2). As shown by the phylogenetic tree generated with the X-*ComEC* amplicon sequence variants, different *Xylella* lineages could be separated based on sequence differences, with Xt representing the most distinct group from the three Xf clades (Figure 3). Using Sanger sequencing on the X-*ComEC* PCR products, we were able to confirm the identity of 22 *Xylella* bacterial monoculture samples up to species (for Xt) and subspecies (for Xf) level. These results demonstrate that in addition to detection, X-*ComEC* PCR can be used for *Xylella* species and subspecies differentiation.

3.4 | X-ComEC PCR specificity and sensitivity

The limit of detection of the X-*ComEC* PCR was determined through a TPS as per the EPPO PM 7/122 (2014) protocol across five diagnostic laboratories that used different PCR reagents and equipment. Using the X-*ComEC* PCR, the five participating laboratories generated consistent trends in detection limits for the dilution series of all four representative isolates of different *Xylella* linages. The detection limit typically ranged from 87.54 to 875.4 DNA copies/μL, with some detections at 8.754 DNA copies/μL (Figure 4a). The detection limit of the Xf qPCR developed by Harper et al. (2010) was comparable to that of X-*ComEC* PCR in all dilution series with the exception of Xt. The X-*ComEC* PCR performed similarly for both Xf and Xt; in contrast, the Xf qPCR by Harper et al. (2010) could not detect Xt. The use of different PCR reagents or thermocyclers had no apparent effect on the sensitivity of the X-*ComEC* PCR assay, nor did there appear to be any PCR bias towards amplification of specific lineages. Results of two TPS participants demonstrated that the detection limit of this PCR could be extended by increasing the cycle number to 40 cycles (Figure 4b).

3.5 | Nanopore sequencing for fast turnaround species and subspecies identification

Two nanopore sequencing runs (nanopore sample test and nanopore sensitivity test) were performed to test the compatibility of nanopore sequencing technology with X-*ComEC* PCR identification.

In the nanopore sample test run, approximately 450,000 reads (211.75 Mb) were generated after 2 h and 21 min of sequencing. After preprocessing and demultiplexing, an average of 25,535 reads per barcode passed the quality filtering. Using these reads as input, all three consensus calling methods produced correct and valid species and subspecies identification for most of the tested samples (Table 3). The BLASTn result of the consensus sequences generated by the reference-guided method had the highest nucleotide identity and query coverage, indicating that the consensus sequence generated by this method is the most accurate. The de novo sequence cluster method generated correct identification without the need for a reference sequence, despite the lower percentage identity and query coverage compared with the other methods. The reference-guided method appeared to be the most sensitive method as it was the only method to provide a correct identification for the Xf *Vitis* stock sample that had a low read count. However, it is noteworthy that the result from this sample was regarded as indeterminate because it had a lower N50 value than the cut-off for a valid identification.

Results from the nanopore sensitivity test run provided more insight on the detection limit of nanopore sequencing. In this run, approximately 1.74 million reads (804.6 Mb) were produced in 20 h and 23 min of sequencing. We observed differences in read counts and mapping rate from samples that had detectable positive bands (PB samples) and those that were undetectable (NTC and NB samples; Table 4). The PB amplicons generated more than 20,000 pass reads and more than 95% of these reads aligned to the X-*ComEC* PCR amplicon sequences. Amplicons of NB samples generated approximately 1000 to 3000 pass reads with less than 10% mapping rate. In comparison, fewer than 1000 reads were detected in the NTC samples. Compared with NTC and NB samples, the N50 value of the PB samples (487–550 bp) was also higher and closely resembled the expected amplicon size (650 bp). The accuracy of the three consensus calling methods in subspecies and species identification were also compared (Table 4). The de novo sequence cluster method could correctly identify the *Xylella* samples at species and subspecies level for 22 out of the 23 PB samples, whereas the reference-guided method identified all PB samples correctly. In contrast, the Geneious method could not distinguish between Xfp and Xff in eight PB samples. Also, 11 of the identifications produced by the Geneious method did not meet the query coverage cut-off and were classified as indeterminate results.

Although only identification derived from PB samples should be considered valid, we have taken our investigation further onto the NB samples to assess the detection limit of nanopore sequencing. We asked whether or not nanopore sequencing can identify *Xylella* correctly from low read-count (or low bacterial titre) samples that would otherwise be undetectable on gel electrophoresis. Our results showed that the subspecies and species identification results were mostly incorrect for NB samples. The de novo sequence cluster method could only correctly identify seven out of 17 NB samples and it produced two misidentifications and no detections for 10 samples, including the NTC. The reference-guided method misidentified 11 out of 17 NB samples and, of more concern, it produced a Xfm identification for the NTC samples. The Geneious method did not generate misidentification for any of the NB samples or NTC samples. These findings show that *Xylella* lineage identification is unreliable when samples have no-band on gel electrophoresis, low read count or low N50.

4 | DISCUSSION

Xylella spp. are recognized globally as some of the most important plant-pathogenic bacteria scientifically and economically (Mansfield et al., 2012). While Xf has emerged as a devastating pathogen globally, Xt is a relatively lesser-known pathogen that has only been observed infecting Nashi pears in Taiwan. To ensure accurate and reliable *Xylella* detection and monitoring in case of an outbreak, an effective molecular test suitable for both *Xylella* species is essential. Although various molecular tests have been developed specifically for the detection of Xf, it is unclear whether these tests are equally effective for detecting Xt. Currently, only a PCR assay and a qPCR assay are capable of detecting both Xf and Xt (Ito & Chiaki, 2021; Ito & Suzaki, 2017). The development of diagnostic methods for the universal detection of both *Xylella* species lags behind. To bridge this knowledge gap, this study presents a new diagnostic assay—X-*ComEC* PCR—that can not only identify *Xylella* species but also determine the subspecies of *fastidiosa* through amplicon sequencing.

The X-*ComEC* PCR primer set was designed by leveraging a large dataset of publicly available *Xylella* genomes and a complementary set of 'outgroup' genomes of closely related bacterial species. Considering both the specificity towards *Xylella* and the unwanted nonspecific binding with closely related bacterial species, this genome-informed approach generated the X-*ComEC* PCR primer set design with 100% homology to *Xylella* genomes. Comparing to three other published conventional PCR tests, the X-*ComEC* PCR primer set is the only primer set that has no mismatches between primers and all *Xylella* genomes (including all subspecies of Xf and Xt). Our study identified several cases of primer–template mismatches in three other published conventional PCR tests. The primer mismatches we identified in these *Xylella* primer sets were not located at the 3′ end of the primer sequences and therefore generally considered to have minimal effect on PCR efficiency. However, previous studies of other PCR targets such as 16S rRNA PCR had shown that internal primer–template mismatches in various locations could negatively affect the thermal stability of the annealing process, and thus reduce the PCR specificity (Bru et al., 2008; Kwok et al., 1990; Stadhouders et al., 2010). Furthermore, only X-*ComEC* PCR and the X67S1/XL2r primer sets could amplify and detect both Xt and Xf. Overall, our analyses demonstrate that X-*ComEC* PCR is compatible for amplifying all genomes of Xf and Xt.

Interlaboratory TPS and assay validation are essential to the development of diagnostic assays (Cardwell et al., 2018). To establish the performance and limitation of the X-*ComEC* PCR as a *Xylella* diagnostic test, a comprehensive TPS was performed across five national diagnostic laboratories in Australia and New Zealand. An important aspect of PCR assay performance is the diagnostic specificity and sensitivity towards the target pathogen. Belonging to the *Xanthomonadaceae*, *Xylella* is closely related to other *Xanthomonadaceae* including the genera *Xanthomonas* and *Stenotrophomonas* (Naushad & Gupta, 2013). In contrast to the exotic *Xylella* species, many species within the latter genera are commonly found in soil and plants in Australia. Our testing demonstrated that the X-*ComEC* PCR assay detects *Xylella* isolates specifically and did not produce false-positive results with nontarget isolates.

Xylella is known to be difficult to isolate as it requires special culture media to grow on and is very slow growing (IPPC, 2018). An effective molecular test should be able to detect *Xylella* directly from host DNA. Our results indicate that X-*ComEC* PCR is also capable of *Xylella* detection directly from infected plant or insect vector DNA extracts. The cross-reactivity of the X-*ComEC* PCR assay with uninfected plant hosts and their associated microbiome was also considered. The

X-*ComEC* PCR assay did not have nonspecific binding issues with any of the 161 uninfected plant tissue DNA samples extracted from various plant species and geographical origin. Sensitivity testing across all laboratories indicated the limit of detection of X-*ComEC* PCR on Xf DNA samples was 8.754 to 875.4 DNA copies/μL level, which is comparable to the tested detection limit of the Xf qPCR by Harper et al. (2010) (Figure 4a), one of the most recommended molecular assays for *Xylella* diagnostics (EPPO, 2019; IPPC, 2018). Additionally, our results illustrate that the X-*ComEC* PCR outperforms the Xf qPCR by Harper et al. (2010) in Xt detection. Through extensive testing performed in this study, X-*ComEC* PCR was shown to be accurate, highly sensitive and applicable to the detection of both *Xylella* species from bacterial culture, plants and insect samples. However, due to restrictions in importation and handling of live *Xylella* cultures in Australia, the present study could not include limit-of-detection testings using artificial spike-in of live *Xylella* bacterial suspensions with uninfected plant material. Further insect testing would also be required with a broader range of insect species of different geographical origins. Follow-up investigations such as these would be welcomed to assess the full potential of X-*ComEC* PCR.

In recent years, qPCR-based diagnostic assays have become increasingly popular for *Xylella* detection because of their quantitation and real-time detection capabilities. However, given the short length of most qPCR products, Sanger sequencing is not an option without cloning, making conventional PCR a more preferrable and practical diagnostic method for species and subspecies identification. Phylogenetic analysis with the X-*ComEC* PCR indicates that its amplicon sequences are different amongst different *Xylella* isolates at species and subspecies level. Our findings indicate that the PCR amplicons of the X-*ComEC* PCR assay can be used for both detection and identification of *Xylella* lineage by Sanger sequencing or by nanopore sequencing. In addition to this current study, the use of nanopore amplicon sequencing has previously been demonstrated in Xf detection and identification using PCR and MLST (Faino et al., 2021; Marcolungo et al., 2022). In contrast to these previous works, this current study took both *Xylella* species into consideration and demonstrated that X-*ComEC* PCR could detect and differentiate between Xt and various Xf subspecies. On the other hand, similar to these published methods, our findings showed that a nanopore sequencing workflow using a MinION could greatly improve the efficiency of *Xylella* identification. In our experience, the nanopore sequencing workflow from PCR purification (20 min), library preparation (1 h), sequencing data generation (2 h) to data analysis (30 min) could be completed in approximately 4 h. This nanopore sequencing workflow is significantly faster than Sanger sequencing, allowing a fast turnaround time in cases such as an emergency response to an incursion event, although the costs for nanopore sequencing kits (from US $599), flowcells (US $900) and sequencing devices (from US $1000) might make nanopore sequencing less accessible than Sanger sequencing for laboratories with limited resources. The ongoing costs for nanopore sequencing could be lowered by multiplexing and simultaneously sequencing multiple samples in a flowcell. We demonstrated that pooling together 42 barcoded samples in our sensitivity test run generated more than enough data for *Xylella* species identification, bringing the sequencing cost down to US $32 per sample (excluding the cost for sequencing device, shipping and other miscellaneous costs). More progressive multiplexing approaches such as dual-barcoding might be applicable to further reduce the cost for nanopore amplicon sequencing by batch sequencing a larger number of samples in one run (Liou et al., 2020). Generally, our results showed X-*ComEC* PCR to be compatible with nanopore sequencing workflow and a rapid and cost-effective diagnostic methodology for *Xylella* identification.

As X-*ComEC* PCR species and subspecies differentiation relies on detection of SNPs on the *ComEC* gene, the relatively high error rate of nanopore raw reads could potentially lead to incorrect and misleading *Xylella* lineage assignment. To minimize this risk, consensus sequences were constructed using the nanopore raw sequence reads to improve the overall accuracy. Different bioinformatic methods can be used for generating consensus sequences from the nanopore sequencing reads, but the influence of using the different methods on the accuracy in plant pathogen identification is rarely explored. In this study, we considered three different bioinformatic analysis methods. Geneious Prime is a user-friendly commercial bioinformatics tool that is popular amongst biologists as it has a graphical user interface and does not require scripting knowledge or access to high performance computers. In terms of command-line based tools, in this study, two popular methods were compared. Medaka is a genome-polishing tool developed by Oxford Nanopore Technology that also features variant calling and consensus calling function. The NGSpeciesID workflow developed by Sahlin et al. (2021) uses a reference-free approach to first cluster reads by similarity and form initial consensus sequences, followed by a polishing step to generate the final consensus sequence. Our evaluation showed different consensus calling methods had varying accuracy in species or subspecies identification. The reference-guided method had the highest true positive rate, followed by the de novo sequence cluster method and then the Geneious method. Sensitivity testing results also revealed that accuracy of identification by nanopore sequencing dropped significantly when the read count was low or when the PCR amplification could not be verified in gel electrophoresis. Accuracy of *Xylella* identification with nanopore amplicon sequencing could be influenced by a range of different parameters such as length of reads generated, consensus calling methods and the quality of the BLASTn search result. Considering these parameters, we demonstrate that unreliable identification could be eliminated by using the four criteria we set out for X-*ComEC* PCR sequencing result validation. When applying nanopore sequencing with X-*ComEC* PCR in *Xylella* species and subspecies identification, one should also take careful considerations in line with recommendations made in the EPPO standard (PM 7/151 (1), 2022).

Reliable, effective and efficient diagnostic assays are of paramount importance for the detection and management of *Xylella*-related diseases. X-*ComEC* PCR detects Xf, a highly lethal plant

pathogen, as well as Xt, an understudied lesser-known bacterium under the same genus. The X-*ComEC* PCR assay produced equivalent results to assays recommended in the EPPO/ICCP protocols when different *Xylella* isolates were tested and it did not generate false-positive results from other related bacteria nor from uninfected host tissues. It is also one of the first *Xylella* detection assays with both the capabilities for subspecies-level identification and for use with nanopore sequencing technology. In combination with nanopore sequencing, it will improve primary detection and identification of *Xylella* spp. to inform and facilitate outbreak responses. While most diagnostic tests incorporated in the current EPPO/ICCP protocols and Australian National Diagnostic Protocols were rarely tested on their high-throughput sequencing compatibility during their development, this study filled the gap and presented X-*ComEC* PCR as an example case of nanopore sequencing-compatible *Xylella* diagnostic development ready for incorporation in routine diagnostic practice. The X-*ComEC* PCR has recently been incorporated into the newly updated Australian National Diagnostic Protocol for *Xylella* spp. thereby improving biosecurity of a broad range of Australian plant industries, amenities and the environment. We encourage similar recommendations to be made for incorporation of this assay into EPPO/ICCP or other protocols routinely used in other regions.

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DATA AVAILABILITY STATEMENT

The nanopore sequencing data that support the findings of this study are openly available in NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra under BioProject accessions PRJNA1068273 and PRJNA1068283.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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