

Final Report

Improving preparedness of the Australian horticultural sector to the threat potentially posed by *Xylella fastidiosa* (a severe biosecurity risk)

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Improving preparedness of the Australian horticultural sector to the threat potentially posed by Xylella fastidiosa (a severe biosecurity risk) (MT17006)

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Public summary

Xylella fastidiosa is the number one exotic National Priority Pest for Australia due to its broad host range and the significant threat it poses to agricultural and horticulture industries. Outbreaks of *X. fastidiosa* have caused significant crop losses and socio-economic impacts overseas. *X. fastidiosa* is transmitted by insect vectors from one plant to another and can infect over 650 plant hosts. The symptoms caused by *X. fastidiosa* include leaf scorch, chlorosis, and decline and often look like other disorders, such as water stress or nutrient deficiencies. Symptomless infections also occur in tolerant hosts and in sensitive hosts with early or low titre infections and these can act as a reservoir of the bacterium, contributing to its long-term survival and spread in an environment.

The Australian National Diagnostic Protocol (NDP) for *X. fastidiosa* was first prepared in 2010 and updated in 2016. It was focused on Pierce's disease of grapevines, which is primarily caused by *X. fastidiosa* subspecies *fastidiosa*. However, other subspecies occur, including *multiplex* and *pauca*, which are also of economic importance in a broad range of horticultural crops grown in Australia and New Zealand. All subspecies are further described into sequence types. A new species, *Xylella taiwanensis*, which only occurs in Taiwan and only infects *Pyrus pyrifolia* (Asian pear), was also discovered. The different species, subspecies and sequence types can differ in host specificity and can cause a range of mild to severe symptoms or be symptomless, therefore the NDP must include tools to differentiate them during primary detection and identification, to inform the outbreak response and surveillance strategies. In addition, since the NDP was last updated new diagnostic technologies have been developed that can improve reliability for *Xylella* detection. Therefore, in MT17006 "*Improving preparedness of the Australian horticultural sector to the threat potentially posed by Xylella fastidiosa (a severe biosecurity risk)*" the NDP was updated and broadened to encompass primary detection, diagnosis, and surveillance of all *Xylella* species, subspecies, and sequence types across a broad range of hosts. Project participants included Agriculture Victoria Research (AVR, project lead), New South Wales Department of Primary Industries (NSW DPI), Western Australian Department of Primary Industries and Regional Development (DPIRD), Queensland Department of Agriculture and Fisheries (QDAF) and New Zealand Ministry for Primary Industries (NZ PHEL).

The updated *Xylella* spp. NDP will be a key resource for the accurate diagnosis of *Xylella* spp. and for timely management and eradication of this devastating pathogen if it is detected in Australia or New Zealand. The NDP includes a combination of extensively validated laboratory- and field-based molecular assays, that were published by other international researchers or developed within the project, for the identification and differentiation of *Xylella* spp. down to sequence type and for use during high throughput surveillance. High throughput sequencing and rapid multilocus sequence typing methodologies were also developed, to enable accurate identification of *Xylella* species, subspecies, and sequence type to inform surveillance strategies based on host specificity. MPI developed a collection of accurately characterized *Xylella* spp. isolates, and DNA was shared with the Australian project participants; this critical resource not only assisted the NDP development but will support future *Xylella* spp. diagnosis and surveillance within Australia and New Zealand. This collaborative approach to developing and validating a world leading *Xylella* spp. NDP has strengthened the diagnostic and surveillance capabilities for Australia and New Zealand and contributed to the preparedness of both countries in case of an incursion of *Xylella*. The updated NDP for the detection of *Xylella* spp. has been submitted to the Subcommittee for Plant Health Diagnostics (SPHD), Plant Health Australia (PHA), and will be made publicly available once it is endorsed.

Technical summary

This Hort Innovation project MT17006 addressed the following activities to update the National Diagnostic Protocol (NDP) for *Xylella* spp.:

Activity 1: Establish a collection of *X. fastidiosa* isolates – live cultures and DNA

Activity 2: Improved diagnostics for *X. fastidiosa* to subspecies level and further to sequence typing

Activity 3: Develop rapid in-field diagnostic capability for *X. fastidiosa*

Activity 4: Update the current Australian National Diagnostic Protocol for *X. fastidiosa* to include subspecies identification and establish diagnostic capability in major node diagnostic laboratories in Australia

Activity 5: Conduct proficiency testing activities to ensure technical staff in participating Australian diagnostic laboratories are trained and proficient in technical procedures listed in the *X. fastidiosa* NDP

Summary of Activities

Activity 1: Establish a collection of *X. fastidiosa* isolates – live cultures and DNA

- Vouchered specimens of at least 10 *X. fastidiosa* established in at least one of the collaborating laboratories in Australia and/or New Zealand

A collection of 22 vouchered *Xylella* spp isolates, including 21 *X. fastidiosa* and one *X. taiwanensis*, were imported as cultures (glycerol stocks) into New Zealand by Ministry for Primary Industries (NZ PHEL) and DNA was extracted for use in Australia And New Zealand. DNA of the 22 vouchered *Xylella* spp. isolates is housed by Agriculture Victoria Research (AVR) and New South Wales Department of Primary Industries (NSW DPI). DNA from a subset of isolates was also provided to Western Australian Department of Primary Industries and Regional Development (DPIRD) and Queensland Department of Agriculture and Fisheries (QDAF). Leaf tissues of 21 infected plant samples were imported from the USA by NZ PHEL and DNA was extracted. All DNA were distributed to the project collaborators for use in the testing, validation, and optimisation of diagnostic assays for the *Xylella* spp. NDP.

An inventory has been populated with genome assemblies (97 strains) publicly available on the National Center for Biotechnology Information (NCBI), the 22 vouchered cultures obtained by NZ PHEL and the 19 *Xylella* spp. infected plant samples from USDA. Five of the 22 vouchered strains, which were curated in International Collection of Microorganisms (ICMP) and the 21 USA plant samples were sequenced in this project. Sequence typing was done on all NCBI downloaded genomes, all vouchered strains and imported plant samples. This information would serve as primary source to check sequence types of available strains of *X. fastidiosa*. The inventory also contains a list of DNA from *Xanthomonas* sp. and *Stenotrophomonas* sp. isolates held by NSW DPI that were used in the specificity panel for test validation and information about the *Xylella* spp. dilution panel that was used to test assay sensitivity. The inventory can be used to assist users of the NDP to identify materials to establish assays and find resources that aid detection and identification of *Xylella* spp.

During this project importation of live *Xylella* cultures into Australia was attempted by Dr. Toni Chapman (NSW DPI) to aid in the development and delivery of molecular diagnostics tools in the NDP for detection of the bacterium. However, importation of live cultures of National Plant Priority Pests (NPPPs), which includes *Xylella* spp, is strictly regulated due to the perceived risk to industry and a complex process of approvals is required to obtain an import permit from the Department of Agriculture, Fisheries and Forestry (DAFF). The application to import live cultures and specimens that contain viable *Xylella* spp. into Australia has been approved under strict conditions and cultures will be imported as part of the European travel component. Although our New Zealand neighbours will retain cultures, it is imperative that Australia also has independent access to a supply of cultures to support ongoing diagnostic development and to ensure continuity of local access to positive controls in case of an outbreak.

Activity 2: Improved diagnostics for *X. fastidiosa* to subspecies level and further to sequence typing

- Literature review on laboratory assays for *X. fastidiosa* and its subspecies.
- Evaluate identified protocols from the literature; determine if these are suitable for sub-speciation.
- Develop genome-informed diagnostic assays, if required.
- Generate SOPs for inclusion in the NDP and distribute to collaborating labs in Vic, WA, Qld and NZ labs for inter-laboratory evaluation and training.
- Field sampling and validation of the SOPs under Australian conditions.
- Training session held in collaboration with Vic which will focus on both the laboratory and the in-field diagnostic assays.
- Visit the USA (citrus, grapevines) and Europe (almonds, olives, summer fruit) to gain first-hand practical experience on sampling procedures for *X. fastidiosa*.

Literature review identified numerous PCR assays for detection of *Xylella* spp. at various taxonomic levels along with gaps where published assays did not meet the needs of an Australian NDP (e.g. generic tests). Many of the assays were initially trialled against panels of *Xylella* spp. and closely related species but did not demonstrate sufficient specificity and were rejected. New, genome-informed PCR assays were developed in the project to meet these needs using publicly available genomes and genome generated in the project. Finally, six PCR assays, including two genome-informed assays developed in the project, were rigorously evaluated using the dilution and specificity panels and by testing a broad range of plant hosts collected throughout Australia and New Zealand for off target detection. Based on consistency of detection amongst the project partners as well as specificity and sensitivity, the following assays are recommended for primary detection of *Xylella* spp. and incorporated in the draft NDP:

- For samples other than Asian pear use the Harper et al (2010, erratum 2013) qPCR assay in combination with a generic endpoint PCR assay developed in this project.
- For Asian pear samples use the Duplex qPCR assay which combines the *X. taiwanensis* specific qPCR (developed for this project) and Harper et al (2010, erratum 2013) qPCR assays, and the endpoint PCR assay developed in this project.

Sensitivity testing indicated that the qPCR assays were more sensitive than the endpoint PCR assays and therefore they are recommended as the primary screening tests. However, the use of the generic *Xylella* spp. endpoint assay benefits the diagnostic workflow because it enables confirmation of detection of both *Xylella* spp. and three known *X. fastidiosa* subspecies by Sanger or nanopore sequencing of the resulting amplicon. Nanopore sequencing enables rapid inhouse *Xylella* spp. identification using Geneious software for analysis of a small number of samples or the reference-free NGspeciesID method for a larger number of samples. For these reasons, the generic *Xylella* spp. endpoint PCR is recommended as the confirmatory test on any suspect positive samples. High throughput laboratory screening for surveillance can be done using the Harper et al (2010, erratum 2013) qPCR assay for *X. fastidiosa* and the specific qPCR designed in this project for *X. taiwanensis*.

Although new assays were developed for in-field detection of *Xylella* spp. (Activity 3, see below), it was demonstrated that the Harper et al (2010, erratum 2013) loop-mediated isothermal amplification (LAMP) assay can also be used as a *Xylella*-generic confirmatory test in the laboratory.

Multilocus sequence typing (MLST) using seven genes was adopted from the literature and coupled with Oxford Nanopore Technology (ONT) sequencing to enable rapid identification of *Xylella* species, subspecies and sequence types. ONT-MLST was demonstrated to have 10-fold greater sensitivity than the qPCR assays.

A whole genome sequencing capability for characterising *Xylella* spp. was established using short read sequencing on an Illumina instrument and ONT sequencing using a MinION device. Procedures to *de novo* assemble draft genomes for identification of the subspecies and sequence types using MLST approaches and the published core genome multilocus sequence analysis (MLSA) pipeline UBCG (Na et al 2018) were established. This activity has given the participating laboratories experience and enabled them to establish a capability for *Xylella* spp. identification and characterisation using HTS approaches. Therefore, whole genome sequencing for *Xylella* spp. identification and characterisation is listed in the NDP workflow, although the specific HTS methodology is not given.

SOPs were written for all PCR, LAMP and MLST methodologies for incorporation into the NDP.

Travel to Brazil, USA and Europe is being undertaken to ensure that Australian scientists meet with overseas experts and gain direct experience in field identification of *Xylella* spp. based on symptoms, gather information about sampling

strategies for various hosts and the methodology used by overseas colleagues for primary and high throughout laboratory and field-based detection. Travel is being undertaken in May and June 2023:

- Dr Pragya Kant (AVR) is travelling to Brazil and the USA, 21st May-5th June and will meet with the following international experts:
 - Dr Helvécio Della Coletta-Filho, Instituto Agronômico de Campinas, Centro de Citricultura Sylvio Moreira, Cordeirópolis, SP, Brazil
 - Prof Joao Lopes, Department of Entomology, University of Sao Paulo, ESALQ/USP, Piracicaba, SP, Brazil,
 - Dr Lindsey Burbank and colleagues at USDA, SJVASC Parlier, California, USA
 - Dr Rodrigo Almeida and colleagues at University of California, Berkeley, USA.

At the time of submission of the final report Dr. Kant had completed the first week of her travel and the summary of findings is provided in Appendix 4.

- Dr. Toni Chapman (NSW DPI), Dr. Monica Kehoe (WA DPIRD) and Dr. Luciano Rigano (NZ PHEL) are travelling to Italy, Spain and France 29th May – 24 June and will meet with the following international experts:
 - Dr. Blanca Landa, Institute for Sustainable Agriculture, CSIC, Alameda del Obispo S/N, Córdoba, 14004 Spain (travel in Italy and Spain)
 - Dr Sophie Cesbron and Dr Marie Agnes-Jaques, INRAE, Angers, France
 - Dr Anne-Laure Boutigny, Anses, Plant Health Laboratory, Bacteriology Virology GMO Unit, 7 rue Jean Dixméras, 49044 Angers cedex 01, France
 - Dr Perrine Portier French Collection for Plant Associated Bacteria, Institut Agro, INRAE, Univ Angers, IRHS, SFR QUASAV, CIRM-CFBP, F-49000 Angers, France.

The findings from the visit to expert laboratories and field may result in further updates of in the *Xylella* spp. NDP and will provide new images of *Xylella* spp.-infected hosts for the NDP. Additional preserved positive control material will be obtained to further support the development and application of the NDP. Learning about vector movement and containment will inform monitoring and surveillance strategies for *Xylella* spp. The final contribution will be to provide the learnings of these trips at a workshop on the NDP for *Xylella* spp., to be held before the end of 2023.

Activity 3: Develop rapid in-field diagnostics capability for *X. fastidiosa*

- Conduct a literature review of current *X. fastidiosa* in-field diagnostics, identify methods to evaluate.
- Evaluate identified protocols and validate.
- Optimise sampling procedures from both plants and insects, including sensitivity assays to determine the possibility of pooling samples.
- Generate SOP of validated in-field protocols for inclusion in the NDP.

The literature review identified four LAMP assays for *X. fastidiosa* and *X. taiwanensis* detection, including one that differentiates the subspecies *fastidiosa* and *multiplex* (Harper et al 2010, erratum 2013, Yaseen et al 2015, Aglietti et al 2019 Burbank & Ortega 2018). Two generic LAMP assays to detected both *Xylella* spp. were designed within the project. Additionally, a commercial recombinase polymerase amplification (RPA) assay (AmplifyRP® XRT+ for *X. fastidiosa*, Agdia) (<https://www.agdia-emea.com>; Li et al 2016) was also identified. Like the PCR assays, these assays were also rigorously evaluated for specificity and sensitivity using the dilution and specificity panels and by testing a broad range of plant hosts collected throughout Australia and New Zealand for off target detection.

Due to their sensitivity and specificity, the Harper et al (2010, erratum 2013) LAMP and the RPA assays were recommended and included in the NDP. Like the LAMP assays, the RPA assay can be visualized using a portable real-time fluorescence reading device. However, if a reading device is not available, the RPA endpoint detection can be done with a lateral flow device (LFD). Both assays provide a result within 20 minutes which is faster than qPCR (> 1 hours) and are simple to use for adequately trained surveillance staff. SOPs were written for incorporation into the NDP. For in-field surveillance, it was recommended to use the Plant Lysis Kit (OptiGene Limited) for sample preparation prior to testing due to its ease of use for non-specialists. In the laboratory, the DNeasy Plant Mini Kit (DNeasy) is preferred for DNA extraction

as it improves sensitivity.

These in-field assays will be valuable as a triage tool for detection of potential *Xylella* spp. infection in the field. They will also be a valuable triage tool in the lab for urgent samples and can be used as a confirmatory tool as described in the NDP diagnostic workflow. As mentioned above, training in the use of RPA and LAMP technologies was provided to 30 diagnostics and biosecurity staff from organizations across Australia and the 2023 ADSW.

Activity 4: Update the current Australian National Diagnostic Protocol for *X. fastidiosa* to include subspecies identification and establish diagnostic capability in major node diagnostic laboratories in Australia

- Include molecular test SOPs to identify subspecies into the NDP.
- Add section 9 (in field diagnostics) to the NDP.
- Submit to SPHD for evaluation.

The Australian NDP for *X. fastidiosa* has been updated as part of this project, to broaden its focus from Pierce's disease in grapevines to all *Xylella* species, subspecies and sequence types that infect and cause diseases in a broad range of crops and other plant species. It includes a diagnostic workflow (Figure 1) based on a range of methodology that was adopted or developed, evaluated, validated, and verified by the project partners. PCR is the primary method for detection and the NDP also includes isolation and culturing, resampling of the specimens, the use of LAMP assays, subspecies-capable assays and sequencing (single amplicon, MLST and whole genome) for confirmatory testing to support primary detection and for identification of species, subspecies and sequence types to support a response. The combination of these methods reduces the risk of false positive results and enable accurate diagnosis of *Xylella* spp. The final version has been submitted to the NDP coordinator at Plant Health Australia for review and endorsement by SPHD. All assays recommended in the *Xylella* spp. diagnostic workflow are established at AVR, DPIRD, NSW DPI, NZ PHEL and QDAF.

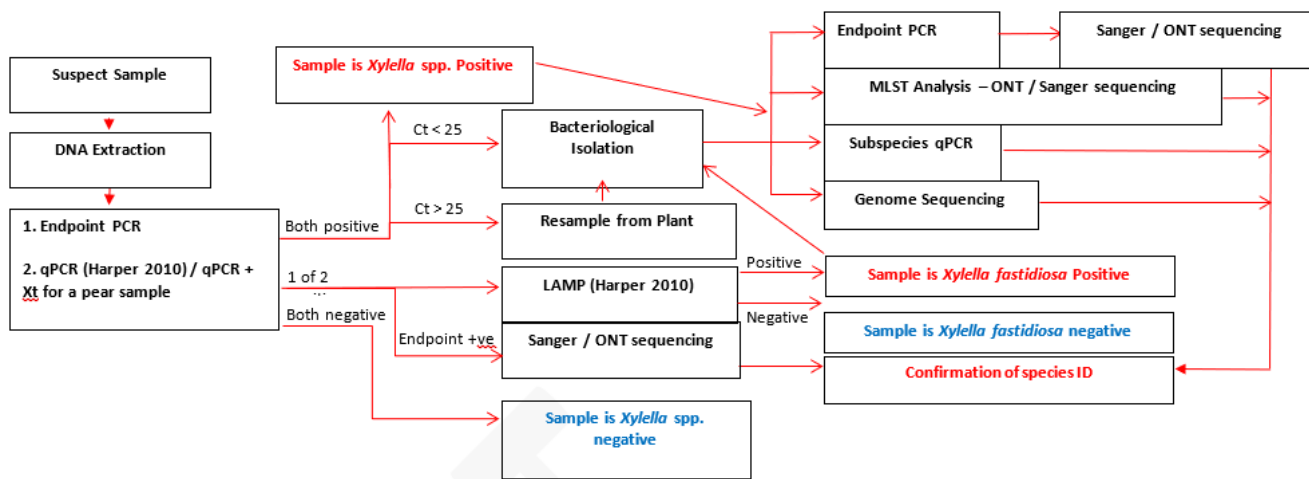


Figure 1. The diagnostic workflow recommended in the updated Australian National Diagnostic Protocol (NDP) for primary *Xylella* spp. detection, identification, and confirmation.

Activity 5: Conduct proficiency testing activities to ensure technical staff in participating Australian diagnostic laboratories are trained and proficient in technical procedures listed in the *X. fastidiosa* NDP

- Generate samples (culture, DNA) for distribution to participating labs.
- Identify labs within Australia and New Zealand who want to participate in the PT program.
- Engage ANQAP to run PT program.
- Conduct PT program with participating labs.

Proficiency testing demonstrated the capability and preparedness of the AVR, DPIRD, NSW DPI, NZ PHEL and QDAF laboratories to perform a *Xylella* spp. diagnosis using the Duplex qPCR and the endpoint PCR combined with sequencing of the amplicon. Each laboratory was able to correctly detect *Xylella* spp. from two blind testing panels of DNA in two rounds of proficiency testing. The first panel consisted of DNA from three naturally infected *Xylella* positive plant samples and three *Xylella* negative plant samples. The second panel consisted of DNA that was extracted from ten plant samples that were spiked with various DNA extracted from *Xylella* spp. cultures.

Five users across AVR, DPIRD, NSW DPI, NZ PHEL and QDAF demonstrated the capability to analyse raw sequence data from four *Xylella* infected samples and one uninfected sample as a control using the StringMLST (Gupta et al 2017), Blastn and NGspeciesID analyses developed in this project. All users correctly identified the negative control and the sequence type of the four *Xylella* samples using all three bioinformatics analysis methods.

Additional Activities Achieved during this project

Dr. Robert Taylor and Dr. Toni Chapman attended the workshop 'Plant diseases caused by *Xylella fastidiosa*: Detection, identification, monitoring and control', Zaragoza (Spain), 12-16th November 2018. The learnings from this workshop were invaluable to this project, all workshop material was made available to the project team and contacts were made which is enabling the international travel which is planned for this project in May/June 2023.

Dr. Robert Taylor and Dr. Toni Chapman were selected as part of the drafting group to author the revision of IPPC diagnostic protocol (DP)25: *Xylella fastidiosa* (2021-003). The lead author of the group is Sophie Cesbron (INRAE France), and co-authors are Toni Chapman (NSW DPI, Australia), Helvecio Della Coletta Filho (CCSM-IA, Brazil) and Bruno Legendre (ANSES, France). The Discipline Lead is Robert Taylor (MPI, New Zealand) and Referee is Brendan Rodoni (AVR, Australia). This IPPC protocol has now been updated and the final version sent to the discipline lead Robert Taylor for acceptance and resubmission to the IPPC for approval.

Based on this project NSW DPI and AVR have been accepted to participate in a Euphresco project on the 'Diagnosis of *Xylella fastidiosa*: detection on dormant plants, important for Mediterranean countries 2202-A-406'. Further funding is being sought to develop this capability in Australian laboratories, through the DAFF Biosecurity Innovation Program.

The project team have delivered numerous presentations and workshops as part of this project, to update on project activities, present project outcomes and to provide training in diagnostics elements of the NDP. The activities are detailed below.

Keywords

Xylella fastidiosa; *Xylella taiwanensis*; National Diagnostic Protocols (NDP); diagnostics; surveillance; molecular assays; polymerase chain reaction (PCR), real-time PCR (qPCR), loop-mediated isothermal amplification (LAMP); recombinase polymerase amplification (RPA); high-throughput sequencing (HTS); multilocus sequence typing (MLST); species; subspecies; vector; horticulture; vouchered specimen.

Introduction

Xylella fastidiosa, a bacterium transmitted by common sap-sucking insects such as spittlebugs and sharpshooters, is one of the most harmful plant pathogens worldwide. The pathogen is not yet present in Australia and New Zealand and is considered Australia's Number 1 National Priority Plant Pest. *X. fastidiosa* can cause significant disease on a number of agriculturally important crops to Australia and New Zealand including grapevines, almond, citrus, stone fruit, blueberries, cherries, dried fruit, macadamias, lucerne and olive, as well as numerous ornamental hosts such as oak, elm, maple and oleander.

Over 650 cultivated and uncultivated herbaceous and woody plant species are known hosts of *Xylella* spp. (664 species; Delbianco et al 2022). The host range is anticipated to increase as different subspecies of *X. fastidiosa* invade new territory (IPCC 2017; Hopkins and Purcell 2002; Chatterjee et al 2008) and potential new vectors are realized. Detection can be difficult with the pathogen having a long latent period and not all plant hosts will exhibit symptoms. All of these factors can contribute towards a long period between pathogen introduction, detection, identification and a biosecurity response. This highlights many risk pathways for Australian and New Zealand biosecurity agencies to monitor, a difficult task given that little is known about how our native plants and invertebrates could respond or contribute to the pathogen's spread if an introduction was to occur.

In Puglia, Southern Italy in 2013, a subspecies of *X. fastidiosa* caused a serious outbreak on olives with some regions losing 100% of their historic olive groves. This outbreak was caused by *X. fastidiosa* subsp. *pauca*, a subspecies previously not known to infect olives, and the pathogen is likely to have been imported into the country via infected ornamental plant material from South America. Initially it was thought that the insect vector of this pathogen was likely to be a leaf hopper, as is the case for other *X. fastidiosa* subspecies such as *X. fastidiosa* subsp. *fastidiosa*. However, spittle bugs, which are endemic in mainland Europe and considered to be inconsequential vectors, were the major contributor to the spread of the disease (Cornara et al 2017).

Strains of *X. fastidiosa* differ in pathogenicity and can behave like different diseases in terms of host specificity and the presence or absence of an efficient insect vector. For this reason, it is imperative that Australia and New Zealand considers the different *X. fastidiosa* species and subspecies associated with different diseases and different crops, especially those on key commercial crops, as it will inform the surveillance response in the event of an outbreak. Fast and accurate diagnosis of *X. fastidiosa* subspecies and strains directly from plant tissue will be critical in an incursion response and all species, subspecies and strains need to be prevented from entering Australia.

There are two described species within the genus *Xylella*: *X. taiwanensis* and *X. fastidiosa*. *X. taiwanensis* causes pear leaf scorch disease on *Pyrus pyrifolia* (Asian pears) in Taiwan and was proposed as a novel species in 2016 (Su et al 2016). The classification of *X. taiwanensis* as a new species is based on genetic and phenotypic differences between *X. taiwanensis* and strains of *X. fastidiosa*.

Different subspecies of *X. fastidiosa* are described in the literature (Denancé et al 2019):

- *X. fastidiosa* subsp. *fastidiosa* is the causal agent of Pierce's disease on grapevine and infects a large range of host plants including coffee, peach, alfalfa, citrus, cherry and almond.
- *X. fastidiosa* subsp. *multiplex* causes scorch disease of many plant species including olive, myrtle-leaf milkwort, peach, pecan, apricot, plum, blueberry and almond.
- *X. fastidiosa* subsp. *pauca* infects citrus, coffee, olive, myrtle-leaf milkwort, cherry and almond.
- *X. fastidiosa* subsp. *sandyi* causes oleander leaf scorch.
- *X. fastidiosa* subsp. *morus* is associated with mulberry leaf scorch occurring in the eastern USA and California.
- *X. fastidiosa* subsp. *tashke* has been isolated from the ornamental tree *Chitalpa tashkentensis* in the United States.

Recent studies have consolidated *X. fastidiosa* into three subspecies: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *pauca*. Within the *X. fastidiosa* subsp. *fastidiosa* group is *X. fastidiosa* subsp. *fastidiosa* sensu largo, which contains the previous subspecies *sandyi* and *morus* (Marcelletti and Scortichini 2016). *X. fastidiosa* subspecies *tashke* is rare and there is no isolate or genome information, therefore its taxonomic status is uncertain.

Subspecies of *X. fastidiosa* are vectored by a range of insects including leafhoppers and spittle bugs. Little is known about

the competency of native Australian invertebrates to vector the various subspecies of the pathogen on important agricultural crops or native hosts.

Diagnostic assays are needed for the direct detection of *X. fastidiosa* in plants as disease symptoms are non-diagnostic for all host species and the pathogen has fastidious growth requirements meaning that isolation of the bacterium onto media can take up to 30 days. Symptoms are too generic and can be confused with water stress and other diseases. The current international protocol for differentiation of subspecies of *X. fastidiosa* is a multilocus sequence analysis (MLSA) using PCR and Sanger sequencing of seven genes (IPPC, ISPM 27, 2018). This approach can be used on DNA extracted from either bacterial cultures or infected plants tested positive for *X. fastidiosa*, however for amplification of DNA direct from plant tissue, it has been observed that the quality of the target DNA may not always be suitable for obtaining all amplicons, or the occurrence of possible mixed infections, may prevent clear assignment of subspecies. In addition, this method is not only time consuming, meaning slow turnaround times for diagnostic results, but it is also costly.

Over the years, several PCR assays have been designed to differentiate subspecies and even strains of *X. fastidiosa*. However, outside the use of MLSA, there is no validated protocol to rapidly distinguish the subspecies of *X. fastidiosa*. There is a need, using a genomics approach to investigate the development of sensitive, specific and robust molecular assays that can quickly detect and identify *X. fastidiosa* subspecies.

This project is part of the larger Hort Innovation project MT17006 Improving preparedness of the Australian horticultural sector to the threat potentially posed by *Xylella fastidiosa* (a severe biosecurity risk) and addresses the following research and development priorities:

- Build capacity to ensure early and accurate detection of *X. fastidiosa*
- Reduce potential impacts on plant movement within, and from Australia.

The three specific objectives are:

1. High throughput tests that differentiate between sub-species and genetic strains (considering the potential for in-field diagnostic tests)
2. Consideration of sampling procedures for both symptomatic and non-symptomatic hosts
3. Capacity to undertake emergency diagnostic response for industry.

To address the objectives this project will:

1. Establish a collection of *X. fastidiosa* isolates – live cultures, DNA and genome sequences
2. Improved diagnostics for *X. fastidiosa* to subspecies level
3. Develop rapid in-field diagnostics capability for *X. fastidiosa*
4. Update/replace the current Australian National Diagnostic Protocol (NDP) for *X. fastidiosa* to include subspecies identification and establish diagnostic capability in major node diagnostic laboratories in Australia.
5. Conduct proficiency testing activities to ensure technical staff in participating Australian diagnostic laboratories are proficient in technical procedures listed in the *X. fastidiosa* NDP.

These research activities align with recommendations from the international symposium on *X. fastidiosa* that was run by Department of Agriculture, Fisheries and Forestry (DAFF) in Brisbane in May 2017 (<https://www.agriculture.gov.au/biosecurity-trade/pests-diseases-weeds/plant/xylella/international-symposium-xylella-fastidiosa>). Based on the experiences associated with *X. fastidiosa* outbreaks in Europe in recent years it was recommended that: (i) access to *X. fastidiosa*-positive controls in containment is critical and required to support best practice diagnostics, (ii) testing and improving media to culture the bacteria be on-going, (iii) development of diagnostics tests, including to the level of subspecies identification and for high throughput detection, is required and (iv) a capability to rapidly characterise a subspecies and/or strains at a genetic level is essential to support effective biosecurity response.

This project is a collaboration between Agriculture Victoria Research (AVR, project lead), New South Wales Department of Primary Industries (NSW DPI), Western Australian Department of Primary Industries and Regional Development (DPIRD), Queensland Department of Agriculture and Fisheries (QDAF) and New Zealand Ministry for Primary Industries Plant Health & Environment Laboratory (NZ PHEL). The Australian collaborators on this project represent four major diagnostic labs in Australia that currently provide diagnostic capability to Australian horticultural industries and to state and national biosecurity agencies and NZ PHEL collaborators represent the national reference laboratory located at the Plant Health and Environment Laboratory (PHEL). Each organisation has significant experience in endemic and exotic phytopathogenic bacteria in temperate, subtropical, and tropical horticulture, including *X. fastidiosa*.

Activity 1: Establish a collection of *X. fastidiosa* isolates – live cultures and DNA.

Fast identification of subspecies of an *X. fastidiosa* isolate that is intercepted in either country is critical to inform a measured and accurate incursion response – not all *X. fastidiosa* subspecies affect all plant hosts. By identifying the subspecies quickly and accurately, surveillance programs can identify appropriate containment of infected plants, based on potential susceptibility of the identified subspecies. Vouchered specimens are required within Australia and New Zealand to support *Xylella* spp. diagnostics and biosecurity responses in Australia and New Zealand.

Genomics approaches to support the development of accurate and robust molecular assays is a proven strategy (Mann et al 2013) and there is a requirement to sequence more strains of *X. fastidiosa* at a global level to improve our understanding of the diversity and prevalence of sub-species and sequence type to inform risk and management. The information also assists in ensuring the specificity and reliability of diagnostics assays.

Methodology

Specific activities will include:

1. Obtain permit for importation of *X. fastidiosa* cultures and/or DNA (for this project we need live cultures to support proficiency testing activities)
2. Establish links globally and import relevant isolates for access to and supporting development of quality molecular assays (lab and field based).
3. Where needed sequence genomes of *X. fastidiosa* strains of interest.

At a minimum it is anticipated that vouchered specimens of up to 10 isolates for the major *X. fastidiosa* subspecies will be established in Australian diagnostic labs. The project team will also aim to establish DNA and sequence data for up to 30 *X. fastidiosa* isolates (10 isolates per year) over the course of the three-year project. Obtaining these positive controls will also be crucial during analysis of subspecies type during an incursion or interception at the border to determine whether the detected isolate is a novel recombinant or an already described sequence type.

A major risk of this Activity is a limited or inadequate import permit for DNA and live cultures of *X. fastidiosa*. This is critical but is also contingent on current DAFF policy. The research team identifies this issue as a major risk factor for this project. NZ PHEL and NSW DPI applied to the relevant regulatory authorities in each country for permits to import live cultures of *Xylella* spp. and infected plant materials to aid in the development and delivery of molecular diagnostics tools in the NDP for detection of the bacterium and support diagnostics capability in both countries. The participating laboratories developed an agreed register of *X. fastidiosa* strains to source for the project. The main criteria used when selecting strains was to achieve representation of all *X. fastidiosa* subspecies from different geographic origins, and to also include *X. taiwanensis*. NZ PHEL established links with key research groups in Europe (Euphresco; CIHEAM) and the USA (USDA-APHIS) to identify and obtain cultures of key *Xylella* spp. isolates and *Xylella*-positive plant material from a range of hosts.

This research project will work in collaboration with international partners to strategically sequence *X. fastidiosa* isolates and where possible place the sequence data in a central database for access by all researchers. This database of *X. fastidiosa* genomes will inform development of targeted molecular diagnostic activities (see Activity 2). The sequencing and bioinformatics capability at AgriBio (AVR) will be used to sequence isolates of interest for the project team. This will also ensure that Australia and New Zealand are prepared and skilled to quickly sequence any *X. fastidiosa* isolates that are intercepted at our borders.

By generating genome sequences, the partner laboratories are using and developing the optimal procedures for sequencing a fastidious xylem limited bacterium. These wet lab procedures and bioinformatics approaches used in “peace time” will be invaluable for these diagnostic labs if *X. fastidiosa* is intercepted in Australia and a quick identification is required. This capability will be established in several labs in Australia and at the NZ PHEL. This capability will be essential during an incursion with the ability to call on several laboratories in both New Zealand and Australia to handle the surge in sample numbers for testing.

Results and discussion

NZ PHEL obtained permits and regulatory approvals to import and house live cultures and plant material infected with *X. fastidiosa*. The following materials were obtained (milestones 103, 104, 105):

- Total of 22 live cultures, including all *X. fastidiosa* subspecies and *X. taiwanensis*, were obtained from British collection National Collection of Plant Pathogenic Bacteria (NCPBP) United Kingdom (n = 4), French Collection of Plant-Associated Bacteria (CBFP) France (n = 12) and International Collection of Micro-organisms from Plants (ICMP, n = 6).
- Dr Lindsey Burbank, a Research Scientist from the US Department of Agriculture in California submitted a set of three different grape cultivars (Scarlet Royal, Thompson seedless, Flame seedless), that had been artificially infected with *X. fastidiosa* subsp. *fastidiosa* strain Stag's Leap. A non-infected Thompson seedless control sample was also provided.
- Dr. Burbank provided two additional grapevine samples.
- Dr Sebastian Albu, a plant pathologist from the California Department of Food and Agriculture submitted a set of field-collected *Xylella*-positive plant samples from different hosts including olive, oleander, and grape.

The live cultures were obtained as glycerol stocks and were successfully cultured by NZ PHEL on growth media recommended in the NDP for *Xylella* spp. isolation and culturing. *X. fastidiosa* was also cultured from a fresh grapevine sample. DNA was successfully extracted from cultures and plant samples (milestones 103, 104, 105).

DNA was from infected plant tissue and insect samples suspected to be infected with *Xylella* was provided by Department of Agriculture, Fisheries and Forestry and is held at DPI NSW for use in diagnostic validation and evaluation. NSW DPI have created a culture collection of closely related species in the *Stenotorophomonas* and *Xanthomonas* genera (Appendix 2), which occur in Australia, and extracted DNA for evaluation of the molecular diagnostic assays included in the National Diagnostic Protocol (NDP) for *Xylella* spp.

A database of *Xylella* spp. sequences was created by whole genome sequencing of the six ICMP isolates imported by NZ PHEL and DNA of nine isolates from infected plant tissues or cultures that were previously obtained by AVR. The database also includes publicly available genomes that were downloaded from the NCBI database. Isolates in the database have also been sequence typed using bioinformatics pipelines (Appendix 2). Appendix 2 has been created and each tab has stains used in this project.

During this project importation of live *Xylella* cultures into Australia was also attempted to aid in the development and delivery of molecular diagnostics tools in the NDP for detection of the bacterium and, as of May 2023, is still being progressed by NSW DPI. Importation of live cultures of National Plant Priority Pests (NPPPs), which includes *Xylella*, is strictly regulated due to the perceived risk to industry and a complex process of approvals is required to obtain an import permit from the Department of Agriculture, Fisheries and Forestry (DAFF). Roadblocks to importation can include the availability of appropriate levels of containment, waste disposal, and the perceived risk of escape and spread associated with storing, handling and movement between laboratories of live cultures and samples containing viable organisms. The difficulties in importing *Xylella* and other NPPPs experienced by Australian research institutes and diagnostic laboratories led to several national workshops on the importation and domestic movement of viable organisms for diagnostics that was organised by the Subcommittee on Plant Health Diagnostics (SPHD). At the first workshop DAFF presented a revised importation process (<https://bicon.agriculture.gov.au/BiconWeb4.0/>; [Importing plant research material - DAFF \(agriculture.gov.au\)](https://bicon.agriculture.gov.au/BiconWeb4.0/)), under which NSW DPI applied to import *Xylella*. However, for *Xylella* and other NPPPs it is still critical to gain industry acceptance for importation. Consequently, specific meetings were held in December 2022 and February 2023 at which provided information on the importance of the work in this project and the need to import live *Xylella* cultures to support biosecurity systems for Australian agricultural industries was presented to biosecurity leaders in DAFF, state jurisdictions and industry. The DAFF Plant Import Operations team also toured the NSW DPI Quarantine Containment 3 (QC3) facility at the Elizabeth Macarthur Agricultural Institute (EAMI) where the current application outlines that isolates would be stored and used. The application is in the final evaluation process and if successful, NSW DPI will also import *Xylella* spp. cultures and infected samples to support biosecurity preparedness in Australia and New Zealand.

One of the primary aims for the importation of *Xylella* is for use as a positive control for culturing the organism, which is an important diagnostic process in the NDP. It enables biosecurity preparedness of the laboratories by ensuring the reagents and media used for isolation and culturing of the bacterium are of sufficient quality and aids evaluation of shelf-life limitations. Having live cultures of the different species, subspecies and sequence types is important to ensure Australian diagnosticians gain practical experience in the correct preparation of the three different media used for isolation, learn how to culture, and can differentiate *Xylella* spp. from other phyto bacteria on the media as long as they

can access the QC3 facilities at EAMI or have their own QC3 facilities and permits. As an additional activity, these cultures can be used to generate Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (MS) profiles as a diagnostic tool for confirmation of *Xylella* spp. after isolation. This tool detects and identifies signature molecules that can be used for rapid bacterial identification from isolated colonies. The method has not been included in the current NDP because live cultures were unable to be imported into Australia during the life of the project. However, if importation is successful signature molecule libraries will be generated and shared as part of the MALDI-TOF standards working group that has been formed as part of a SPHD/DAFF activity.

Another benefit of having live cultures is that they can be used to continually generate a source of DNA to support molecular diagnostic development, for diagnostic testing of suspects samples and during monitoring and surveillance. Extraction of nucleic acid is a critical step in ensuring reliable detection of *Xylella* spp. by molecular methods. In this project DNA extraction could only be evaluated in Australia by spiking in purified *Xylella* spp. DNA provided by NZ PHEL into homogenates or DNA extracts of uninfected hosts and this was done using citrus, grapevine olive, which are some of the plant species occurring within the top ten host families for *Xylella* spp. (Table 1). However, this does not provide a true appraisal of extraction efficiency as it does not consider the ability of the method to release cells from the xylem and lyse the bacterial cells to release DNA. This is process of evaluation is critical for detection of *Xylella* spp. as it infects a broad range of hosts includes 88 families, 299 genera, 664 species (Delbianco et al 2022), some of which are more difficult to extract nucleic acid from than others. Therefore, having live cultures that can be used to spike samples at different bacterial concentrations, or having samples infected with viable organisms, is needed to assist in evaluating the efficiency of DNA extraction methods for detection of *Xylella* spp. in a range of hosts and host tissues using molecular techniques as well as evaluating the true efficiency of specific molecular tests. This is also required as a part of laboratory ring and proficiency testing, to ensure all Australian and New Zealand laboratories are capable of extracting quality DNA from a range of hosts for *Xylella* spp. detection. These would be evaluated using the primary PCR methods that are included in the NDP for *Xylella* spp. detection, identification and surveillance.

Activity 2: Improved diagnostics for *X. fastidiosa* to subspecies level

This activity is targeted towards the development of an updated NDP that can be applied to a broad range of hosts, including almonds, summer fruit, citrus, olives, blueberries, cherries, macadamias, coffee, and ornamental species, and can identify the different species and subspecies of *Xylella*. The development of the updated NDP will build on the current work being undertaken by Rob Taylor (NZ PHEL PHEL), who is working on a *Xylella fastidiosa* diagnostic protocol for the IPPC. Through this collaboration, plus the knowledge, skills and pipelines developed as part of the Plant Biosecurity CRC project PBCRC2156, the key objective will be to develop diagnostic assays capable of subspecies identification for *X. fastidiosa*, under Australian conditions. Once these assays are optimised they will be transferred to all project team members for validation and for inter-laboratory consistency.

Additional methods for inclusion in the NDP include culturing of *Xylella* spp., determining what selective medias are available and if there is any variation between the different subspecies in growth requirements and isolation. Another important factor is to determine how selective the medias are for *Xylella*, given there are a number of endemic Xanthomadaceae family members including *Xanthomonas* and *Stenotrophomonas*, plus any native plant bacteria which may confound isolation during an incursion.

Methodology

1. Conduct a literature review of current *X. fastidiosa* diagnostic assays, identify methods to evaluate the gaps in diagnostics for *X. fastidiosa* and its' subspecies.
2. Protocol development and evaluation.
 - Evaluate identified protocols from the literature; determine if these are suitable for sub-speciation.
 - Develop genome-informed diagnostic assays, if required. It is likely that subspecies specific molecular based assays will need to be developed.
 - Generate SOPs for inclusion in the NDP and distribute to collaborating labs in Vic, WA, Qld and NZ labs for inter-laboratory evaluation and training.
 - Field sampling to be conducted in NSW, Vic, WA and Qld for testing and validation of the SOPs under Australian conditions.

- Develop/Update the National Diagnostic Protocol for *X. fastidiosa* and submit to SPHD for evaluation and ratification
3. Training session which will focus on both the laboratory and the in-field diagnostic assays. This training activity will be made available to all interested parties in Australia and New Zealand.
 4. Visit the USA (citrus, grapevines) and Europe (almonds, olives, summer fruit) to gain first-hand practical experience on sampling procedures for *Xylella fastidiosa*.

A comprehensive literature review was carried out to determine the known species and subspecies of *Xylella*, their hosts and current distribution. The current methods to detect and identify *Xylella* species, subspecies and sequence types were also reviewed along with culture isolation, any specific growth conditions for the subspecies from different plant material. As part of the review of molecular methods for *Xylella* spp. detection and identification, DNA extraction from different plant and insect material was also included. Access to publicly available genome sequences was assessed as this information was required to support diagnostic assay development, particularly at the subspecies level.

Isolation: Due to the ability to import live cultures and plant material infected with viable organisms, NZ PHEL were able to test culturing procedures that are included in the NDP. They also evaluated a DNA extraction procedure for the molecular detection and characterisation of *Xylella* spp.

Sequencing: High throughput sequencing (HTS) methods were established to enable draft genomes of *Xylella* spp. to be obtained from culture (whole genome sequencing) and plant samples (metagenomic sequencing). A total of 24 isolates were sequenced, including the six ICMP *Xylella* isolates for which genome data had not been previously obtained. The method used Illumina short read technology and the sequence data was analysed by *de-novo* assembly to assemble draft genomes. Bioinformatic analyses used to identify species and sequence types were a published core genome MLSA pipeline UBCG (Na et al. 2018) as well as several analyses that enable sequence typing by MLST (Scully et al 2005) including *de-novo* assembly and web-MLST, mapping reads to multi-locus sequence typing (MLST) markers and web-MLST and using the software tool meta-MLST (Milestone 106).

An alternative and rapid approach to sequence typing was also developed using which combines PCR amplification of MLST genes and nanopore sequencing of the amplicons using a MinION device (Oxford Nanopore Technologies) (Milestone 105, 106 and 108). Seven house-keeping gene partial products *cysG* (600 bp), *holC* (379 bp), *gltT* (654 bp), *leuA* (708 bp), *malF* (730 bp), *nuoL* (557 bp) and *petC* (533 bp) were amplified from key *Xylella* spp. isolates using the MLST primers of Yuan et al. (2010) according to Faino et al. (2019). For each sample amplicons of each gene are purified, quality checked, quantified and then pooled in equal concentration for library preparation and then sequenced using the MinION. Bioinformatic analyses to assign sequence type were done using with StringMLST (Gupta et al 2017), MiniMap2 (Li 2018), which maps raw reads to an *X. fastidiosa* MLST allele database (from <https://pubmlst.org>), BLAST (Altschul et al 1990) and NGSPECIESID (Sahlin et al 2021). A standard operating procedure (SOP) was developed for MLST analysis, including wet and dry lab work.

PCR: The specificity and sensitivity of 14 PCR assays (quantitative and endpoint) identified in the literature and assays developed within this project was evaluated using a total of 81 samples including strains of *X. fastidiosa* from culture, *X. fastidiosa* infected insect hosts, *X. fastidiosa* infected and uninfected plant tissues Assays were also evaluated against *Xanthomonas* spp. and *Stenotrophomonas* bacteria which occur in Australia and New Zealand and are closely related to *Xylella* spp. to test for off target detection. Evaluation of DNA extraction procedures on symptomatic and asymptomatic tissue of key host species was also conducted (Milestones 104 and 105).

All project partner laboratories undertook verification and validation testing for the selected qPCR, endpoint PCR, LAMP assays and the MLST bioinformatic analysis to further evaluate methods and to facilitate establishment of the assays in all laboratories. Validation used a range of DNA that was extracted from the 22 *Xylella* spp. cultures, 17 *Xylella* positive plant samples, 4 *Xylella* positive insect samples, 15 *Xanthomonas* spp. and eight *Stenotrophomonas* bacteria (Appendix 2). Each of the partner laboratories also evaluated the assays against a sensitivity panel comprising of a dilution series for each of *X. fastidiosa* subspecies (*X. fastidiosa* subsp *fastidiosa* ICMP8731; *X. fastidiosa* subsp *multiplex* ICMP8739; *X. fastidiosa* subsp *pauca* CFBP8072 and *X. taiwanensis* NCPPB4612). DNA of each isolate was diluted to a stock concentration of 2.4 ng/μl. These stock concentrations were then supplied to all participating laboratories for the assay evaluation. Each participant diluted the isolate DNA to a final concentration of 2.4 x 10⁻¹⁴ ng/μl. These were then tested in triplicate using each assay. The assays were also used to test a total of 403 plant samples spanning 49 genera and which were collected across Australia (including Norfolk Island) and New Zealand (Appendix 1). The detail of all the plant samples and their origin was provided in Milestone 108 Table 1.

Project partners provided training to stakeholders in the form of presentations on the development of methodology and the NDP and training on the MLST, RPA and LAMP methodologies, in partnership with Plant Health Australia, at the Annual Diagnostics and Surveillance Workshop, Canberra, 9-11 May 2023.

Travel to gain first-hand practical experience on sampling procedures for *X. fastidiosa* was organised for May and June 2023:

- Dr Pragya Kant (AVR): Brazil and the USA, 21st May-5th June
- Dr. Toni Chapman (NSW DPI), Dr. Monica Kehoe (WA DPIRD) and Dr. Luciano Rigano (NZ PHEL): Italy, Spain and France 29th May – 24 June.

Results and discussion

1. Literature review

The aim of the review was to ensure Australian and New Zealand laboratories had access to an updated NDP that contains world's best practice diagnostic methods for the detection, identification, monitoring and surveillance of *Xylella* spp. Reviews of the literature were included in Appendix 1 of Milestones 103 and Milestone 104. Even after these reviews were completed the project team ensured they remained up to date with new publications, which enabled further identification of diagnostic approaches that could be developed within the project for inclusion in the NDP and to build capability and solid knowledge base amongst the project collaborators.

The review identified the currently known and optimal sampling strategies for detection of *Xylella* spp. in several hosts by culture isolation and molecular methods and this information was used to update the NDP sampling methodology to maximize the likelihood of detection. Symptomatic material collected in triplicate at moderate temperatures provides greatest chance of *Xylella* spp. detection. Limited research is available to guide sampling and testing of asymptomatic plants, where titre of *Xylella* spp. is likely to be lower. There is also limited information about sampling dormant plants and plants in tissue culture. A Euphresco project on the 'Diagnosis of *Xylella fastidiosa*: detection on dormant plants, important for Mediterranean countries 2202-A-406' will commence in 2023 and AVR and NSW DPI have been accepted to participate and funding is being sought to evaluate the Australian NDP on dormant tissues and tissue cultures as part of this collaboration.

Based on current research both the EPPO (European Plant Protection Organization) and the IPPC (International Plant Protection Consortium) protocols for detection of *X. fastidiosa* consider molecular tests the most sensitive for screening of symptomatic plant material (IPPC, 2018; EPPO, 2018). Therefore, methods such as ELISA, which can offer a simple and high throughput approach to testing to facilitate monitoring and surveillance, were not chosen for evaluation. ELISA also requires intact bacteria, although they can be inactivated, as positive controls and because Australia does not have a permit to import cultures of *Xylella* spp. it would not have been possible to evaluate this method.

As part of a preliminary gap analysis, a review of the literature and discussion amongst the team identified that diagnostic testing should target sequence subtypes (ST) within each *X. fastidiosa* subspecies, because they can have different host ranges. Therefore, subtyping approaches were developed for inclusion in the NDP to facilitate targeted surveillance during an incursion.

The review also highlighted the importance of whole genome sequencing to support molecular diagnostics development, bacterial characterization, biology and ecology and traceability. This supported the use of whole genome sequencing to characterize *Xylella* spp. isolates in the project.

Numerous molecular assays have been published for *Xylella* spp. detection, but not all are suitable for a more broad-based detection of the different species, subspecies and sequence types required in the NDP. Therefore, after careful assessment, a total of three endpoint PCR assays, seven qPCR assays, two subspecies PCR assays were identified for evaluation in the project. It was also decided to develop a generic *Xylella* spp. endpoint PCR assay, a generic qPCR assay and a specific *X. taiwanensis* qPCR assay.

A Multilocus Sequence Typing (MLST) assay was also identified from the literature to enable *X. fastidiosa* sequence typing. (Milestone 103 and 104). MLST for determination of sequence type of the *X. fastidiosa* is based on amplification and sequencing analysis of seven housekeeping genes. Based on the variance in each gene an allele number is assigned for that gene. MLST is officially adopted as the international standard for the identification of *X. fastidiosa* sequence types as outlined in the EPPO and IPPC diagnostic protocols (Scally et al 2005, Yuan et al 2010). The seven house-keeping genes

used were *cysG* 600 bp, *holC* 379 bp, *glT* 654 bp, *leuA* 708 bp, *malF* 730 bp, *nuoL557* bp, *petC* 533 bp. Knowing sequence types is very important because there is an association between sequencing analysis and host specificity, and it can inform the evolutionary relatedness of the isolates for traceability. The final SOP is included in Appendix 5 (this report).

In addition to the laboratory-based methods described above, five assays that can be used in the field and in the laboratory for *Xylella* spp. detection were identified for evaluation, including four LAMP assays and one RPA assay. (Milestone 103 and 104).

2. Protocol evaluation and development

Isolation:

The culturing and sampling procedure were adopted from EPPO /IPPC protocols and incorporated into the NDP. NZ PHEL successfully detected *Xylella fastidiosa* in petioles of infected grapevine material (Figure 2) supplied by Dr Burbank (USA) using qPCR and by isolation on buffered charcoal yeast extract (BCYE) media (Figure 3) at PHEL (Milestone 104). This significant, “hands-on” experience working with plant tissue infected with *X. fastidiosa* enabled:

- Development and validation of a protocol for isolation of *X. fastidiosa* from infected plant tissue.
- Validation of a method for purification of high-molecular weight DNA from infected plant leaf petioles.
- Addition of a new strain to the project’s vouchered specimen collection that will allow distribution of purified DNA to the broader project team for genome sequencing and other downstream applications.



Figure 2: *Xylella fastidiosa* infected grape samples received by NZ PHEL from Dr Lindsey Burbank, which were used for evaluation isolate and culturing methods and molecular methods for *Xylella* spp. detection.



Xylella fastidiosa ICMP8745 on BCYE. Dr Luciano Rigano MPI NZ

Figure 3. *Xylella fastidiosa* growth on (buffered charcoal yeast extract) agar

Whole genome sequencing and analysis

Whole genome sequencing was identified as a useful confirmation, characterisation, and identification tool for *Xylella* species, subspecies and sequence types (Figure 4, Figure 5). Draft genomes of 24 isolates were *de novo* assembled and were used to identify the subspecies and sequence types using MLST approaches and a published core genome MLSA pipeline UBCG (Na et al 2018; Appendix 2). Some of the voucher specimens were not previously genome sequenced, including six ICMP strains, were identified to subspecies or sequence type level (Appendix 7). These sequences have been incorporated into the genome database that was generated in this project and which is held by all project partners. Databases of *Xylella* genomes, reference genomes and information of sequence types were created and made available to all collaborative laboratories as primary source of information to facilitate test development and strain identification. Although the specific HTS methodology is not given in the NDP, it is given as an option as part of the overall workflow to identify species, subspecies and sequence type to inform a response and support traceability and genomic epidemiology for pathway risk analysis during an outbreak. It can be used to investigate genes and gene function of isolates that exhibit unusual biological or ecological characteristics.

This activity has established a capability in *Xylella* spp. genome sequencing and analysis and the genome data generated in this project was used to inform the development of three new assays, one endpoint PCR, Generic qPCR and Duplex qPCR for *Xylella* spp. detection. The NDP has added genome sequencing as a tool for accurate strain identification as it is useful to support high-resolution traceability.

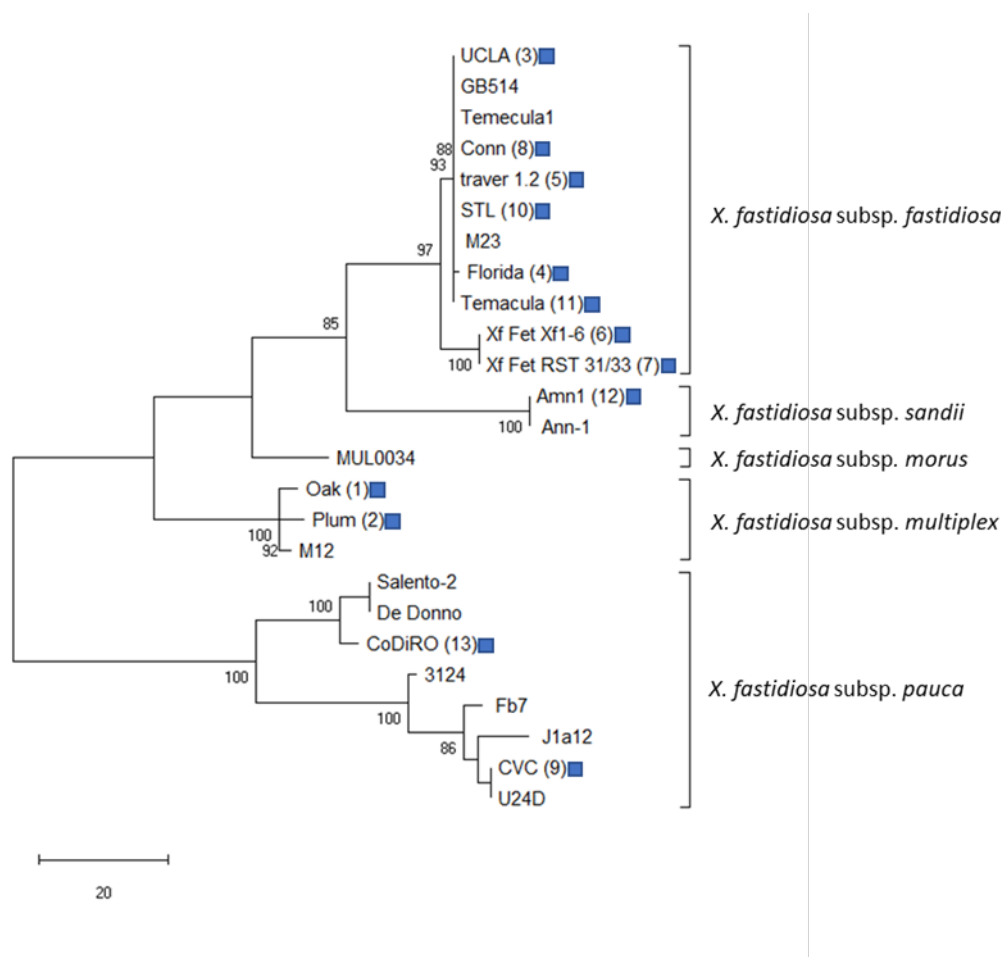


Figure 4. Maximum Parsimony analysis of seven concatenated partial MLST genes (*leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL* and *gltT*) totalling 3,630 bp for the discrimination of *Xylella fastidiosa* strains. DNA positive controls held by AVR are defined by a blue square. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.



Figure 5. MLST relationships of *X. fastidiosa* assemblies that were generated in this project or from data available on NCBI inferred by core genome MLSA pipeline UBCG. DNA positive controls held by AVR are defined by a filled shapes with a triangle indicating *X. fastidiosa* subsp. *fastidiosa*, a circle indicating the former *X. fastidiosa* subsp. *sandii* (now *X. fastidiosa* susp *fastidiosa*), a square indicating *X. fastidiosa* subsp. *multiplex* and a diamond indicating *X. fastidiosa* subsp. *pauca*. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.09994319 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite.

Detection of *Xylella* spp. by PCR

A review of the literature identified numerous molecular assays for *Xylella* spp. detection and identification (Milestone 103). Not all PCR assays were suitable for detection of the various *Xylella* species and subspecies due to specificity issues, therefore five qPCR assays and two generic endpoint assays for detection of *Xylella* spp. were developed (Milestone 105 & 106). These primer sets were tested *in silico* for their specificity for *Xylella* spp. and only one endpoint generic PCR assay and the generic qPCR assay were selected for further development and optimisation. The amplicon of the generic endpoint PCR can be sequenced to identify both species and all subspecies of *X. fastidiosa*. This was demonstrated by phylogenetic comparisons of the sequences of the amplified gene region that were extracted from 97 *X. fastidiosa* and 32 *X. taiwanensis* that were publicly available on NCBI (Milestone 107 Figure 3). The generic qPCR assay combines generic primers with two probes, one specific for *X. fastidiosa* and one specific for *X. taiwanensis*. It was demonstrated that the *X. taiwanensis* specific probe could be used in an independent specific assay or used in a duplex setting with Harper et al (2010, erratum 2013) qPCR to detect both *X. fastidiosa* and *X. taiwanensis*.

After careful assessment of all published and locally developed PCR assays, a total of four endpoint PCR assays, ten qPCR assays, two subspecies PCR assays were evaluated for *Xylella* spp. detection. The assays were rigorously tested against DNA of the 22 *Xylella* spp. cultures, 17 *Xylella* positive plant samples and 4 *Xylella* positive insect samples. They were also evaluated against 15 *Xanthomonas* spp. and eight *Stenotrophomonas* bacteria which occur in Australia and New Zealand and are closely related to *Xylella* spp. to test for off target detection. Only PCR six assays passed the rigorous specificity evaluation and were subsequently evaluated by the project partner laboratories:

- Endpoint generic PCR *Xylella* spp. assay (This project)
- Generic qPCR *Xylella* spp. (This project)
- qPCR for *Xylella fastidiosa* by Harper et al 2010, erratum 2013
- Duplex qPCR for *Xylella fastidiosa* by Harper et al 2010, erratum 2013 and *X. taiwanensis* (This project)
- qPCR for *Xylella fastidiosa* by Ouyang et al 2013
- qPCR for *X. fastidiosa* by Dupas et al 2019

Four qPCR tests showed detection limit ranges between 2.4×10^{-3} to 10^{-6} ng/ μ l for bacterial culture DNA by seven testers in five laboratories (Milestone 107, Appendix 2, Figure1). This testing also confirmed that the limit of detection of *X. taiwanensis* using the specific assays is similar that of the Harper et al. (2010, erratum 2013) qPCR. The comparison with Harper et al. (2010, erratum 2013) assay confirmed that presence of the two probes in the Duplex qPCR does not have any effect on the specificity or sensitivity as the Ct values were not significantly different between the two assay conditions (Milestone 108 Appendix 3, Table 2 and Table 3). The Duplex qPCR using Harper *X. fastidiosa* (Harper et al 2010, erratum 2013) and the *X. taiwanensis* probes showed higher detection limit ranged from 2.4×10^{-4} ng/ μ l to 2.4×10^{-6} ng/ μ l compared to the simplex assays. This assay will be valuable to test pear samples as first detection of *Xylella*, however consistent detection of *X. taiwanensis* was only achieved using the Generic endpoint PCR and Duplex qPCR assay developed in this project.

Sensitivity testing indicated that the qPCR assays were more sensitive than the endpoint PCR assays. However, the use of the generic *Xylella* spp. endpoint assay benefits the diagnostic workflow because it enables confirmation of detection of both species and three known subspecies by Sanger or nanopore sequencing of the 650 bp amplicon.

Testing against the locally collected plant samples from Australia and New Zealand indicate that all assays were specific, except Ouyang (et al 2013) qPCR which provided late CT of 38 and above on uninfected citrus plants and which were negative with all other assays, suggesting a false positive result (Milestone 107 Appendix 4). It is important that assays do not produce late off target detection that could be confused with a low titre infection of *Xylella* spp. and which could lead to a false positive or false negative result and impact on the decision to respond.

Based on consistency of detection amongst the project partners as well as specificity and sensitivity, the following assays are recommended for primary detection of *Xylella* spp. and incorporated in the draft NDP:

- For samples other than pear use the Harper et al (2010, erratum 2013) qPCR assay in combination with the endpoint PCR assay.

- For pear samples use the Duplex qPCR assay combining the *X. taiwanensis* specific qPCR and Harper et al (2010, erratum 2013) assays with the qPCR assay and the endpoint PCR assay developed in this project.

The other assays are recommended as backups to confirm a result in the laboratory and to gain further differentiation, including the Harper et al (2010, erratum 2013) LAMP assay (see activity 3).

Accurate identification of *X. fastidiosa* subspecies and sequence types by amplicon sequencing

A key driver of this project was to ensure that the Australian NDP included rapid and accurate methods to identify *Xylella fastidiosa* to the subspecies level to inform surveillance strategies associated with host specificity. As noted in the project team has determined that the level of identification addressed in this project and the resulting NDP should go beyond *X. fastidiosa* subspecies identification to sequence type because sequence types can have different biology, including disease expression and host range (Milestone 102). Therefore, sequencing approaches were developed for the accurate identification, characterisation and differentiation of *Xylella* species and strain types, and included in the NDP.

One approach is to sequence the generic endpoint PCR amplicon to differentiate between two species and *Xylella fastidiosa* subspecies. Sequencing analysis of the generic 650bp amplicon was tested using three bioinformatics methods to identify *X. fastidiosa* subspecies and *X. taiwanensis* and the Geneious method is recommended when analysing a small number of samples. When working on large numbers of samples, the reference-free NGspeciesID method should be used with a query coverage cut-off at 80%. The amplicons of a negative control (healthy plant DNA) must be sequenced alongside with other samples, even if no amplicon is present when the assay analysed by electrophoresis, as this establishes a baseline on the barcode switching, which could generate false positive results. It is also recommended that the data should be analyzed with multiple approaches for validation purposes (Milestone 108, Appendix 2 Table 2).

To rapidly identify *Xylella* species, subspecies, and sequence type after primary detection, a MLST approach using seven house-keeping genes was adopted from the literature and combined with a nanopore sequencing strategy that was developed in this project. The amplicons of seven genes are generated in individual PCR reactions, pooled and subject to nanopore long read sequencing using a MinION device (method described in Milestone 105, Appendix 2). Three bioinformatic methods to analyse raw sequence reads of the amplicons were evaluated and based on the initial evaluation and proficiency testing amongst the project partners, the StringMLST and BLAST analyses were recommended for the NDP (Milestone 108, Appendix 2 Table 3). MLST has been incorporated into the NDP as a tool to aid decision making for surveillance during a response, as *Xylella* species, subspecies and sequence types can have specific host ranges. MLST combined with MinION nanopore sequencing was shown to be 10-fold more sensitive for detection of *Xylella* species, subspecies and sequence types than the qPCR and LAMP assays by Harper et al (2010, erratum 2013; Milestone 105, Appendix 2 Table 2).

The bioinformatic pipelines developed in this project for MLST analysis are uploaded and are freely available on GitHub page specifically developed for *Xylella* species, subspecies https://github.com/chewbeckie/Xylella_pcrONT and sequence type (https://github.com/Pragya2019/Xylella_MLST_ONT) identification.

Standard operating procedures for PCR and amplicon-based sequencing were developed and incorporated in to the *Xylella* spp. NDP.

Further work is required to develop and evaluate the use of robotics for DNA extraction in comparison to the column-based methods for *Xylella* spp. detection to support laboratory surge capacity during a response. Experience with other pathogens suggests that there may be a negative impact on sensitivity for detection, which is an important consideration for low titer pathogens such as *Xylella* spp. as it may reduce the capacity to pool samples for efficiency and could result on false negative results. However, automation of extraction enables the ability to focus on other activities, thereby increasing diagnostic throughput, which is critical for surveillance in case of an outbreak and monitoring for proof of freedom.

3. Training

Communication via team meetings, the supply of SOPs and the evaluation and validation of the PCR and MLST assays by partner laboratories has ensured that each lab can undertake primary detection using PCR. The labs have established a capability to analyse sequence data generated through amplicon sequencing, MLST or whole genome sequencing *Xylella* species, subspecies and sequence type identification.

Throughout the project, training has extended beyond the partner laboratories through delivery of presentations by the project partners on the development of methodology and the NDP at conferences, workshops, and external meetings.

1. Toni Chapman: *Xylella* spp.: The journey to develop a new National Diagnostic Protocol, APPS 2021.
2. Pragya Kant: Improved diagnostics for *Xylella fastidiosa* through high throughput sequencing, APPS 2021.
3. Toni Chapman: An Update on National Diagnostic Protocol *Xylella* spp. PIC@PEQ Seminar series on 29th of April 2021.
4. Rachel Mann: Diagnostic preparedness for the threat posed by *Xylella* in Australia: Infield detection tools, Plant Biosecurity Research Symposium 2022, in Adelaide, in May 2022.
5. Toni Chapman: The journey to develop a new and updated National Diagnostic Protocols – Key learnings from Citrus Canker and *Xylella* spp. NDP's. NDP online webinar on 23rd November 2022.
6. Pragya Kant: Development of the new NDP for *Xylella* spp identification, biannual meeting between DAFF at Victoria (Mickleham) and AVR staff on 18th July 2022.
7. Pragya Kant: Improved diagnostics for *Xylella fastidiosa* through high-throughput sequencing, Annual Diagnostic and Surveillance Workshop held at AgriBio in Victoria on Wednesday 31st September 2022
8. Pragya Kant: Nanopore in diagnostics, workshop on advances of Molecular diagnostics, September ADSW 2022.
9. Rebecca Roach: Bacterial diagnostics: recent advances, 'ologist workshop on 6 the September 2022, Ecosciences Precinct, Dutton Park.
10. Johanna Wong-Bajracharya: How to be generic but specific? A nanopore sequencing-compatible PCR/qPCR assay for detection and differentiation of *Xylella fastidiosa* and *Xylella taiwanensis*, 9-11 May 2023 ADSW, Canberra.
11. Pragya Kant: Multi-locus target sequencing (MLST) workshop, 9-11 May 2023 ADSW, Canberra.
12. Pragya Kant and Lucy Tran-Nguyen: Field activity preparation *Xylella* NDP: LAMP and Agdia assays, 9-11 May 2023 ADSW, Canberra.
13. Pragya Kant: Targeted sequencing approaches for *Xylella* identification and surveillance, 9-11 May 2023 ADSW, Canberra.

Training on the MLST, RPA and LAMP methodologies was conducted in partnership with Plant Health Australia at the Annual Diagnostics and Surveillance Workshop, Canberra, 9-11 May 2023. There were 30 participants from across Australia, including diagnosticians and biosecurity staff. All presentations are provided in Appendix 7. The participants were given an overview of *Xylella* sequence types and their importance and how it is determined using MLST using nanopore sequencing. The SOP was distributed to all participants. In addition, at ADSW 2023 the LAMP and RPA kits were used for the field activity in the Australian National Botanical Gardens in Canberra, where Section 9 of the NDP was tested. The use of both kits was demonstrated, and the participants had hands-on experience with these assays. Participants tested the LAMP and RPA kits on plants and insect samples that were collected in the gardens. Both the LAMP and RPA kits performed as expected and *Xylella* spp. were not detected in any of the field collected samples. Photos from ADSW 2023 from these activities are included in the report in "Photos/images/other audio-visual material" section

Additional workshops to train diagnosticians in the *Xylella* spp. NDP are planned for later in 2023, including the Australasian Plant Pathology Society conference to be held in Adelaide, South Australia, 20 - 24 November 2023.

4. Travel to Brazil, USA and Europe to gain first-hand practical experience on sampling procedures for *Xylella fastidiosa*.

Travel to Brazil, USA and Europe was planned for years 1 and 2 of this project to enable the project to gain experience in identifying *Xylella* and the associated diseases. It was also an opportunity to obtain preserved infected field positive samples that could be used to further evaluate the molecular assays included in the updated *Xylella* spp. NDP and for proficiency testing amongst the laboratories, to ensure each laboratory has capability in *Xylella* spp. detection. Due to the Covid19 pandemic, travel was delayed until 2023:

AVR travel in May 20- 5 June, 2023:

- Brazil: Visit Dr Helvécio Della Coletta-Filho, who is an expert in *Xylella* and HLB research.
 - Visit citrus orchards with CVC symptoms and olive orchards with OQDS, collect samples and hands-on training for lab methods such as pathogen isolation, culturing and diagnostic protocols.
 - Attend the diagnostic clinic at the Citrus Center Sylvio Moreira, Brazil.
- Brazil: visit Prof Joao Lopes (<http://www.lea.esalq.usp.br/en/lab/laboratory-insect-vectors-plant-pathogens>) who is an expert in vector-transmission of phytophacteria such as *X. fastidiosa* and 'Candidatus Liberibacter sp.'
 - Learn *Xylella* sampling, diagnostics, culturing of both symptom and symptomless plant tissues.
- USA: visit Dr Lindsey Burbank, USDA, SJVASC Parlier, California.
 - Visit Burbank lab and greenhouse research on *X. fastidiosa*
 - Dr. Pragya Kant Seminar at SJVASC center.
 - Meet with Dr. Rodrigo Krugner and tour insectary- entomologist and the research leader of our research unit. He works on glassy-winged sharpshooter and other vectors of *X. fastidiosa*. A lot of his research deals with biological control and mating disruption of insects in agricultural settings
 - Meet with Dr. Chris Wallis - Plant pathologist and chemist who works on *X. fastidiosa* in grapevine as well as a range of fungal pathogens. Much of his research on *Xylella* has been focused on plant phenolic compounds produced in response to infection.
 - Visit Citrus Pest Detection Program with Ray Yokomi- virologist and citrus researcher who has worked extensively on citrus tristeza virus and many other emerging diseases in citrus.
 - Meet with Dr. Jianchi Chen- molecular biologist who has done a lot of work on *X. fastidiosa* genomics and molecular detection. He also works with detection of unculturable pathogens such as Liberibacter/HLB using sequence-based approaches.
- USA: visit Dr Rodrigo Almeida, University of California, Berkeley.
 - Visit southern California where glassy wing sharpshooter (*Homolascus. vitripennis*; GWSS), an invasive insect vector species, has been contained.
 - Meeting with Prof Rodrigo Almeida, Steve Lindow and Sandy Purcell, Alex Kahn, Patrick Lee and Alex Purcell to learn about their projects.

NSW DPI, NZ PHEL and WA DPIRD travel from 29th May – 24 June:

- Italy: visit the olive orchards in Apulia, where *X. fastidiosa* subsp. *pauca* infection is present.
- Spain: meet with Dr Blanca Landa (Institute for Sustainable Agriculture, CSIC, Cordoba)
 - Alicante to see infected Almond trees.
 - Balearic Islands to see numerous infected production hosts and collect samples.
 - One week in Dr Landa's lab to gain experience in isolating *Xylella* from different host plants.
- France: meet with Dr Sophie Cesbron (INRAE, Angers, France) and Dr Marie Agnes-Jaques (INRAE, Angers, France)
 - Corsica to look at native plants and collect samples infected with *Xylella* and collect samples.
 - One week at the University of Angers, Institut Agro, INRAE, Angers, France, to gain experience in methods for isolation using the samples collected in Corsica
- France: Meet with Dr Anne-Laure Boutigny who is the lead on the Euphresco *Xylella* project (Discussed in the additional activities section).

- France: Meet with Dr Perrine Portier who is the curator of the French Collection for Plant Associated Bacteria (CIRM-CFBP) and from which additional *Xylella* spp. cultures will be purchased if an import permit is granted.

Detailed itinerary for travel plans and activities are in Appendix 4.

At the time of submission of the final report Dr. Kant had completed the first week of her travel and the report of findings is provided in Appendix 4. Primary observations include:

- *Xylella fastidiosa* subspecies *pauca* (Xfp) is the primary strain that is present in Brazil and testing is done using a specific qPCR (Oliveira et al 2002) for this subspecies in Citrus, Olive and Coffee.
- Dr. Kant gained experience in *Xylella* spp. Isolation and culturing which is critical since Australian scientists are unable to develop this skill onshore due to the inability to import viable infected material and cultures to support skill development.
- Dr. Kant learned about vector surveillance and identification:
 - Sticky traps and sweep netting are used
 - 27 of the known vectors are specific to olive and a spittlebug, *Clastoptera* sp.1, is dominant
 - 13 known vectors are specific to citrus
- Propagation and maintenance of citrus mother plants under net houses to control Citrus Variegated Chlorosis (CVC) disease to support supply of uninfected buds. This also controls other pests and diseases such as huanglongbing (*Candidatus Liberibacter* sp.), nematodes, *Phytophthora* spp., blackspot (*Guignardia citricarpa*), citrus tristeza virus (CTV) and citrus canker (*Xanthomonas citri* subsp. *citri*).

Activity 3: Develop rapid in-field diagnostics capability for *X. fastidiosa*

On-farm smart surveillance tools provide an opportunity for on-the-spot diagnosis of pests and pathogens. LAMP technology is an emerging on-farm surveillance tool that is easy to use, portable and rapid with test results obtained in less than 45 minutes from the time of sample collection. Several LAMP assays for the detection of *X. fastidiosa* have been developed, including the production of a commercial LAMP assay developed for *X. fastidiosa* to support recent outbreaks of *X. fastidiosa* in Europe. A LAMP protocol is also included in the IPPC diagnostic protocol for *X. fastidiosa*. The *X. fastidiosa* LAMP assay has the potential to be a powerful valuable triage tool in the field during a disease incursion and to provide valuable evidence of absence in real time during targeted surveillance programs.

Significant adaption is required for lab-based LAMP protocols to be used in-field, by field officers, agronomists, and farmers. Sampling and template extraction protocols need to be developed and validated and guidance instructions for interpretation of test results generated in the field needs to be well defined to ensure consistent and reliable interpretation of data. To facilitate fast identification there is also an opportunity to adapt LAMP assays to a real-time assay in the lab to detect *X. fastidiosa* in insect vectors without conducting a DNA extraction. The LAMP assay potentially will be more sensitive due to its ability to handle PCR inhibitors and it could be run on real-time PCR machines in the lab as part of the high-throughput capability.

There are also several other rapid in-field diagnostic tools that are becoming readily utilised in outbreak situations. These include in-field detection with Minion, a portable long read sequencer, and detection with other isothermal amplification kits including recombinase polymerase amplification (RPA). These technologies will be assessed alongside LAMP in the in-field diagnostic component of this project.

Methodology

1. Conduct a literature review of current *X. fastidiosa* in-field diagnostics, identify methods to evaluate.
2. Evaluate and validate identified protocols.
3. Optimise sampling procedures from both plants and insects, including sensitivity assays to determine the possibility of pooling samples.
4. Generate SOP of validated in-field protocols for inclusion in the NDP (Section 9).

A significant gap in the accurate diagnosis of *X. fastidiosa* is sampling protocols from asymptomatic plants, whether using either lab based or in-field diagnostic protocols. AVR and NSW DPI staff will establish links with European colleagues and

identify optimal sampling protocols of asymptomatic and ornamental hosts as well as for key horticultural crops such as almonds, olives and table grapes. All assays were tested using the specificity and sensitivity panels used to evaluating and validating the PCR assays (activity 2).

Validation of the in-field assays under Australian conditions requires testing plants and insects from across Australia. This is critical to ensure nucleic acid extraction procedures are effective in extracting quality DNA with no inhibitors and to test the specificity of the assays and ensure there are no organisms in Australia that may cross react with the assay and cause false positives. This activity does pose a risk for industry. Surveying raises apparent risks of false positives for potential non-pathogenic *Xylella* or closely related bacteria in the Australian environment. For this reason, it is essential that this work is done during the development phase and during 'peace time' rather than during an emergency response. It is important that industry is aware of this possibility and that appropriate support is provided while determining the true identity of the organism.

Results and discussion

1. Literature review

Four LAMP assays for *Xylella* spp. detection were identified in the literature and evaluated, including one that differentiates the subspecies *fastidiosa* and *multiplex* (Harper et al 2010, erratum 2013, Yaseen et al 2015, Aglietti et al 2019, Burbank and Ortega 2018; Milestone 103 Appendix 1). Since none of the published LAMP assays claimed to detect of *X. taiwanensis*, two generic LAMP assays were designed within the project. Additionally, a commercial RPA assay (AmplifyRP® XRT+ for *X. fastidiosa*, Agdia) (<https://www.agdia-emea.com>; Li et al 2016) was also evaluated. Like the LAMP assays, the RPA assay can be visualized using a portable real-time fluorescence reading device. However, if a reading device is not available, the RPA endpoint detection can be done with a lateral flow device (LFD).

2. Evaluation of in -field diagnostic assays

The recommended LAMP (Harper et al 2010, erratum 2013) and the Agdia Amplify RP® XRT RPA assay, using a real-time fluorescence instrument and LFD detection chambers, exhibited 100% specificity when evaluated using the specificity testing panels with amplification of *Xylella* species and no amplification of closely related species ((Milestone 105 Appendix 3). Milestone 107 Appendix 3). Although the subspecies LAMP assay of Burbank and Ortega (2018) for *X. fastidiosa* subspecies *multiplex* as specific for this subspecies, the assay for *X. fastidiosa* subspecies *fastidiosa* was not specific and detected subspecies *fastidiosa*, *multiplex* and *pauca*, therefore it was not tested further and is also not recommended. Out of two LAMP assays designed in the project, one assay which targets a gene encoding nitronate monooxygenase achieved 100% specificity across the specificity panel and *X. taiwanensis* with no cross reactivity, but it was found less sensitive than the Harper (et al 2010, erratum 2013) LAMP assay (Milestone 105 Appendix 3) and was not tested further. When specificity testing of the remaining LAMP assays was done using field collected plants from Australian and New Zealand, the Aglietti et al (2019) LAMP assay intermittently generated late amplification products (>25 mins) from uninfected citrus and grapevine extracts and no template controls, with similar anneal derivative (AD) values to the positive control, indicating the risk of false positive results (Milestone 104, Appendix 4 Table 6). Therefore, its use for *X. fastidiosa* detection was not recommended. The Harper et al (2010, erratum 2013) LAMP assay was further tested on the DNA of field collected native plant samples by all Australian states and New Zealand and no false positive results were reported, further supporting its specificity for *Xylella* spp. (Milestone 107; Milestone 108). Testing of the RPA assays against the DNA field collected plants was not done due to cost associated with the assay.

Initial evaluation of the LAMP assays was done using serially diluted standards made pure *X. fastidiosa* DNA or *X. fastidiosa* DNA spiked into olive DNA (Milestone 104 Appendix 4; Milestone 105 Appendix 4). The LAMP assays of Harper et al (2010, erratum 2013) and Aglietti et al (2019) were most sensitive and detected *X. fastidiosa* at femtogram levels, like the qPCR assays, demonstrating high sensitivity (Milestone 104, Appendix 4; Milestone 107 Appendix 3). The commercial lyophilised LAMP kit, which uses Harper et al (2010), was 10-fold less sensitive when run side by side with freshly made reactions containing equivalent primer concentration for detection of *X. fastidiosa*. Conversely, the Optigene LAMP kit was 10-fold more sensitive in the detection of *X. taiwanensis* compared to freshly prepared Harper LAMP reagents (Milestone 107 Appendix 3). The sensitivity of all LAMP tests and the RPA assays were equivalent for detection of *X. taiwanensis* (Milestone 107 Appendix 3). However, the limit of detection of the serially diluted *X. taiwanensis* was 100 to 1000-fold less than the detection limits observed for the *X. fastidiosa* strains, suggesting that some genetic variation of the species affected amplification efficiency. LAMP and the RPA assays were 10- and 100-fold

less sensitive, respectively, than the recommended qPCR assays for detection of *Xylella* spp. However, they provide a result within 20 minutes which is faster than qPCR (> 1 hours) and are simple to use for adequately trained surveillance staff. They will be a valuable triage tool in the lab for urgent samples and can be used as a confirmatory tool as described in the NDP diagnostic workflow. They will also be valuable as a triage tool for detection of potential *Xylella* infection in the field. The final SOP is included in Appendix 6 (this report).

DNA extraction methods for use with LAMP were also evaluated since co-extracted plant compounds can cause inhibition and reduce assay efficiency for *Xylella* spp. detection. The DNeasy Plant Mini Kit (DNeasy), QuickExtract™ Plant DNA Extraction Solution (Lucigen), a manual method (Yaseen et al. 2015) and the Plant Lysis Kit (OptiGene Limited) extraction protocols (Milestone 105, Appendix 4) were used to extract DNA from Olive, Citrus and Grapevine plant tissues as per the manufacturer's or published instructions. A cytochrome oxidase gene (COX; Tomlinson et al 2010) assay, which is used to evaluate plant DNA quality for use in LAMP, was used to measure the effect of different the extraction procedures on amplification efficiency. The best results were obtained using the DNeasy kit, but this is only suitable for use in a laboratory and will be suitable for both LAMP and qPCR detection of *Xylella* spp. The Plant lysis kit produced the second-best results and was recommended for LAMP detection for *Xylella* spp. because it was easiest to use; it requires minimal to no specialist skills and no optimisation to complete the extraction and is easily field deployable. The use of the Plant Lysis Kit is of great benefit when considering technology transfer to field officers and industry who may not have the experience or the tools to perform more complex methods. However, the plant lysis kit was the most expensive extraction protocol tested in this analysis. The LAMP assays using DNA extracted with the DNeasy kit could be used a rapid laboratory triage tool, since it can provide an initial indication of the presence of *Xylella* spp. while waiting for the results of other assays.

The LAMP and RPA assays can be easily adopted within Australian laboratories, especially where the laboratories are equipped with the Genie II instrument or AmpliFire® Isothermal Fluorometer. These assays can also be done using real-time thermocyclers normally used for qPCR. The RPA assay can be done as an end-point detection method using a LFD. Both assays will enable a rapid preliminary in-field detection, of *Xylella* spp. with any positive samples analyzed within the laboratory for more in-depth identification.

As described in Activity 2, training in RPA and LAMP methodologies was conducted in partnership with Plant Health Australia at the Annual Diagnostics and Surveillance Workshop, Canberra, 9-11 May 2023. There were 30 participants from across Australia, including diagnosticians and biosecurity staff.

Activity 4: Update the current Australian National Diagnostic Protocol for *X. fastidiosa* to include subspecies identification and establish diagnostic capability in major node diagnostic laboratories in Australia

Reviewing and submitting updated NDP to SPHD

The current Australia NDP focuses on *X. fastidiosa* and Pierce's disease of grapevine. Since it was last updated, *X. taiwanensis* was described and greater understanding of the diversity *X. fastidiosa* has been gained, including knowledge of the subspecies and sequence types and their host range. Additionally, because of this knowledge and the detection of *X. fastidiosa* in Europe, significant advances were made in detection and identification methodologies. Therefore, the overall aim of the project was to incorporate validated lab and field-based SOPs into a more generic *Xylella* spp. NDP and submitted to SPHD for endorsement.

Methodology

Specific activities will include:

1. Include molecular test SOPs to identify subspecies into the NDP
2. Add section 9 (in field diagnostics) to the NDP
3. Submit to SPHD for evaluation

Results and discussion

The Australian National Diagnostic Protocol (NDP) for *X. fastidiosa* has been updated as part of this project (Appendix 3, this report), to broaden its focus from Pierce's disease in grapevines to *Xylella* species, subspecies and sequence types that infect and cause diseases in a broad range of crops and other plant species. As part of the recommended workflow (Figure 1) for primary detection, identification, and confirmation of a *Xylella* spp. incursion, the NDP retains the methodology for bacterial isolation, as this is still a critical step in confirming a detection of the *Xylella* spp., and it includes molecular methods developed and validated in this project.

Verification of assays that were developed by one project partner was independently assessed by other project partners over the course of the project to minimize the need for verification of the NDP after submission to SHPD. As a part of the project, DIPRD, MPI NZ and two independent reviewers from AVR diagnostic laboratories reviewed the NDP prior to submission to SPHD. Verification and review during the project will enable a rapid endorsement of the NDP. The NDP was submitted to SPHD in January 2023 and is currently being reviewed by the Diagnostic Protocols Working Group (DPWG). It is anticipated that it will be endorsed by July 2023.

The final version has been submitted to the NDP coordinator at Plant Health Australia for reviewing and endorsement by SPHD. All assays recommended in the *Xylella* spp. diagnostic workflow are established at AVR, DPIRD, NSW DPI, NZ PHEL and QDAF laboratories.

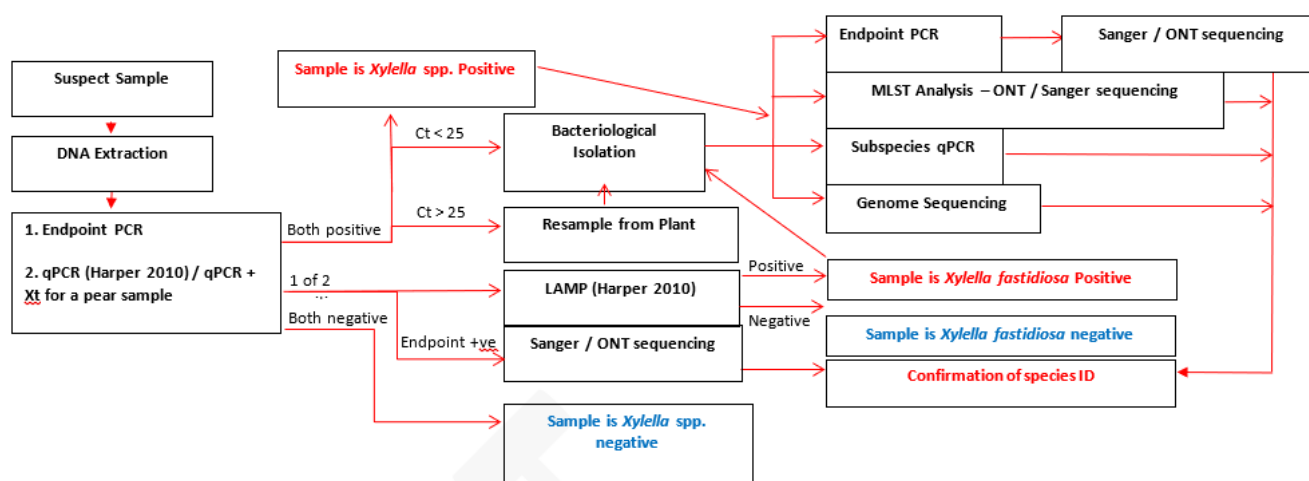


Figure 1. The diagnostic workflow recommended in the updated Australian National Diagnostic Protocol (NDP) for *Xylella* spp. detection, identification, and confirmation.

Activity 5: Conduct proficiency testing activities to ensure technical staff in participating Australian diagnostic laboratories are trained and proficient in technical procedures listed in the *X. fastidiosa* NDP

To ensure that a national diagnostic capability continuously exists for the detection of *Xylella* spp. it is imperative that diagnostic labs in Australia are trained and proficient in running the tests listed in the NDP. To ensure that these protocols are operational, applied consistently, within appropriate laboratory quality systems, and with consistent results within and between laboratories, it is proposed that the diagnostic labs in Australia and New Zealand participate in a proficiency testing (PT) program.

Methodology

The PT program will be run by the Australian National Quality Assurance Program (ANQAP) to ensure independence and confidentiality of test results. The research team will provide test samples to ANQAP for distribution to participating labs. An open call will be placed for participation in the PT program to ensure all interested parties, in addition to this research team, will obtain training and a level of competency in using *Xylella* spp. NDP.

Specific activities will include:

1. Generate samples (culture, DNA) for distribution to participating labs.
2. Identify labs within Australia and New Zealand who want to participate in the PT program.
3. Engage ANQAP to run PT program.
4. Conduct PT program with participating labs.

Results and discussion

Due to the complexities created by the COVID19 pandemic, especially associated with laboratory access during lockdowns, the proficiency testing was not done by ANQAP. Instead, proficiency testing for PCR and MLST was organised within the project (Milestone 108 Appendix 2)

Results of proficiency testing of sequencing-based methods for *Xylella* spp. identification and sequence typing was presented in Milestone 108, Appendix 2. Proficiency testing demonstrated the capability and preparedness of the AVR, DPIRD, NSW DPI, NZ PHEL and QDAF laboratories to perform *Xylella* spp. diagnosis. Two blind testing DNA panels were prepared by NSW DPI for two rounds of proficiency testing that were undertaken by AVR, DPIRD, NZ PHEL and QDAF. The first panel consisted of DNA from three *Xylella* positive plant samples and three *Xylella* negative plant samples. The second panel consisted of 10 samples in which DNA extracted from *Xylella* spp. cultures was mixed with plant material and then the DNA was extracted. These samples were first validated by NSW DPI using the Duplex qPCR (Harper et al 2010 + *X. taiwanensis* developed in the project) and generic *Xylella* endpoint PCR as per the NDP protocol. All participating laboratories achieved 100% accuracy on proficiency testing. Additionally, they were encouraged to identify the species/subspecies ID of these samples by sequencing the endpoint PCR products or performing the qPCRs as described by Dupas et al (2019) for subspecies identification. DPIRD and AVR sequenced the endpoint PCR amplicons using a MinION device. DPIRD analysed data by using the Geneious method with Windows on a computer (Milestone 108, Appendix 2, Table 4) whereas AVR analysed data using the reference-free, command-based Linux system (Milestone 108, Appendix 2, Table 5) Both laboratories accurately detected the subspecies.

Proficiency testing for MLST determination was conducted by dry-lab analysis of sequencing data of five samples including bacterial DNA, Olive DNA spiked with *X. fastidiosa* DNA and metagenomic samples, using the SOPs for StringMLST, Blastn and NGspeciesID analysis developed in this project. Five users across AVR, DPIRD, NSW DPI, NZ PHEL and QDAF achieved correct results, demonstrating the capability within each laboratory to run these pipelines. Sample 5 was a healthy DNA (used as a negative control) and no ST was assigned using all three bioinformatics methods, showing accuracy. All users confirmed correct ST results when using StringMLST and Blastn as expected (Milestone 108, Appendix 2, Table 6).

Additional Activities Achieved during this project

Robert Taylor and Toni Chapman both attended the workshop 'Plant diseases caused by *Xylella fastidiosa*: Detection, identification, monitoring and control', Zaragoza (Spain), 12-16th November 2018. The learnings from this workshop were invaluable to this project, all workshop material was made available to the project team and contacts were made which is enabling the international travel which is planned for this project in May/June 2023.

Robert Taylor and Toni Chapman were both selected to author the revision of IPPC diagnostic protocol (DP)25: *Xylella fastidiosa* (2021-003). The lead author of the group is Sophie Cesbron (INRAE France), and co-authors are Toni Chapman (NSW DPI, Australia), Helvecio Della Coletta Filho (CCSM-IA, Brazil) and Bruno Legendre (ANSES, France). The Discipline Lead is Robert Taylor (MPI, New Zealand) and Referee is Brendan Rodoni (AVR, Australia). This IPPC protocol has now

been updated and the final version sent to the discipline lead Robert Taylor for acceptance and resubmission to the IPPC for approval.

Based on this project NSW DPI and AVR have been accepted to participate in a Euphresco project on the 'Diagnosis of *Xylella fastidiosa*: detection on dormant plants, important for Mediterranean countries 2202-A-406'. Further funding is being sought to develop this capability in Australian laboratories, through the DAFF Biosecurity Innovation Program.

It is anticipated that the travel will lead to an update the diagnostic protocols in the new NDP and provide new images of *Xylella*-infected hosts. Additional preserved positive control material will be obtained and brought back to Australia for the testing of assays incorporated into the NDP. Learning about GWSS movement and containment will inform monitoring and surveillance strategies for *Xylella* spp. The final contribution will be to provide the learnings of these trips at the planned SPHD workshop on the NDP for *Xylella* spp.

Conclusions

In addition to new methodologies developed overseas, this Australian collaborative project has resulted significant technological advances in *Xylella* detection and identification and have been used to update Australian Pierce's disease (*X. fastidiosa*) NDP. Compared to the previous NDP, which focused on Pierce's disease of grapevines, the NDP developed in this project is now broader and is inclusive of both *Xylella* spp., their sub-species and sequence types and is now applicable to a broad range of hosts. The updated NDP has in total eight molecular assays, six for use in laboratories and two for in-field testing. The NDP also includes detailed sampling and culturing details. These additions allow a primary detection to sequence type, which is critical to inform surveillance. The assays will also support surveillance programs during an incursion response or to demonstrate area freedom. Once the NDP is endorsed by SPHD it will become an invaluable diagnostic tool to support the biosecurity of a broad range of plant industries across Australia and New Zealand.

Due to the collaborative nature of this project, four key node laboratories within Australia and the NZ PHEL laboratory have developed a significant diagnostic capability for *Xylella* spp. detection and identification and to support surveillance during an outbreak. This capability is not only built upon the test development, validation and proficiency testing strategy that was undertaken during the project, but also comes from the hands-on experience gained during travel to regions affected by *Xylella* spp. overseas and the associated diagnostics laboratories. Training, through presentations and workshops has created greater awareness in other diagnosticians and biosecurity officers of methodology use for surveillance and demonstrated the applicability of various methodologies for in-field surveillance. Overall, as a result of this project, Australia has a much greater capability to detect, identify and respond to an outbreak of *Xylella* spp. which will help to reduce impact to industry.

Outputs

Table 1. Output summary

Output	Description	Detail
An updated National Diagnostic Protocol (NDP) for <i>Xylella</i> spp. for the use in diagnostic laboratories of Australia and New Zealand	NDP for the detection of <i>Xylella</i> species, subspecies and sequence types have been updated with new assays and met the international standards.	Major node diagnostic laboratories in VIC, NSW, WA, QLD, and NZ have contributed to develop the updated NDP and recommended all assays are in place in these laboratories. The NDP includes six lab-based and two field -based molecular assays. The assays allow for generic detection of <i>Xylella</i> spp. and identification of species, sub-species and sequence types to inform a response. It also includes information on isolation and culturing of <i>Xylella</i> spp. This is evidenced in Milestone 107 appendices 2 and 4 and Milestone 108 appendices 1,2,3. The updated NDP has been submitted to SPHD along with verification and review of the NDP. Once it is endorsed it will be available to all laboratories via the National Plant Biosecurity Diagnostics Network (NPBDN) website.
Build capability of all key node laboratories of Australia and New Zealand to detection <i>Xylella</i> spp.	Conducted proficiency testing activities to ensure technical staff in participating laboratories are well equipped and have resources such and assays primers, protocols and positive controls. Australian diagnostic laboratories personnel are trained and proficient in technical procedures as described in the updated NDP.	To ensure proficiency with the selected assays in the NDP, two blind testing panels were prepared for two rounds of proficiency testing. Initially, three <i>Xylella</i> spp. positive samples and three <i>Xylella</i> spp. negative samples were prepared for assessment with the selected six assays for the NDP. These panels were sent to all partner laboratories for testing, all teams returned the correct results with the testing panel. A second panel was sent to all laboratories, this panel contained 10 samples with DNA mixed with plant material and required each lab to conduct sequencing to determine if they could get the correct species and subspecies. All laboratories returned the correct results. In addition, dry- lab procedures such as bioinformatic scripts analysis were also conducted as apart of proficiency testing to determine the species, subspecies, and sequence types. All laboratories provided correct results. All assays are in place in their laboratories. At the joint Australian Diagnosticians and Surveillance Workshop Canberra, 9-11 2023 a workshop on utility of MLST was delivered to 20 registered participants. LAMP and RPA training for in-field diagnosis of <i>Xylella</i> spp. were demonstrated to 30 participants at the field day workshop.
Vouchered specimens of at least 10 <i>Xylella fastidiosa</i> established in at least one of the collaborating laboratories in Australia and/or New Zealand.	Establishment of the positive controls in New Zealand lab and DNA copies are distributed to all collaborative laboratories in Australia.	Over the course of the project, a significant collection of live cultures of <i>Xylella</i> spp., <i>Xylella fastidiosa</i> infected plant material and DNA from infected insects and plant tissue has been assembled by the project team. The collection includes: - 22 live cultures of diverse <i>Xylella fastidiosa</i> strains of epidemiological relevance from different host species (housed in NZ) - 1 live culture of <i>Xylella taiwanensis</i> (housed in NZ) - 21 samples of <i>Xylella fastidiosa</i> -infected plant material (housed

		in NZ) - 9 DNA extracts from <i>Xylella fastidiosa</i> infected plant samples distributed to laboratories in Australia - 2 DNA extracts from <i>Xylella fastidiosa</i> infected insect vectors This collection of voucher specimens and naturally infected <i>Xylella</i>-positive host material has been vital for the evaluation and optimization of diagnostic methods developed or adapted as part of this project. DNA has been distributed to project partners laboratories in Australia. This is a key resource for the project and to the best of our knowledge, is the most comprehensive collection of <i>Xylella fastidiosa</i> isolates in Australasia. <i>Xylella</i> isolates were successfully cultured, and high-quality DNA extracted from infected plant tissues using protocol described in the NDP. Evidence of establishment of positive control DNA was provided in milestones 104,105, 106 and 107. An Excel sheet is also provided with this report that has details of all the isolates collected and send to all collaborative laboratories for their record.
Implementation of High throughput sequencing in all the collaborating laboratories.	Whole genome sequencing was established in several laboratories for characterization of <i>Xylella</i> spp. from culture Nanopore sequencing capability established to facilitate fast detection of the <i>Xylella</i> spp. and sequence type.	High throughput sequencing on an Illumina platform was used to assemble high quality genomes of five isolates of <i>X. fastidiosa</i> that had not previously been sequenced (Appendix7 of this report) In Milestone 108, Appendix 2 described the method and results of dry lab protocols for species, subspecies and sequence type determination using Illumina and Nanopore sequencing. Github page have been established for <i>Xylella</i> spp. bioinformatics https://github.com/chewbeckie/Xylella_pcrONT. The bioinformatics for determination of sequence type is available at https://github.com/Pragya2019/Xylella_MLST_ONT
Testing native host plant material from Australia and New Zealand with the updated NDP assays.	Complete field sampling of host material as part of the validation of the NDP under Australian conditions.	Milestone 107 Appendix 4 and Milestone 108 Appendix 1 has lists of the samples and the results of testing using the assays included the NDP. No cross-reaction was observed, and no false positive results were detected. In addition, native spittlebugs samples were tested and no positive results this shows that these assays were equally applicable on the insect vectors.
Draft manuscripts	The results from this study have been consolidated for the manuscripts to be published in peer reviewed journals of plant pathology.	Manuscript 1: A novel Nanopore sequencing-compatible generic PCR assay that can detect and differentiate <i>Xylella</i> spp at the species and subspecies level. (Draft in progress) Manuscript 2: The testing and evaluation of quantitative PCR assays for the detection of <i>Xylella</i> spp s in the context of Australian biosecurity. (Draft in progress) Manuscript 3: High-quality full genome assembly of historic <i>Xylella fastidiosa</i> strains from ICMP collection using a hybrid sequencing approach. Appendix 7, submitted to Microbiology) Manuscript 4: Identification of sequence typing of <i>Xylella fastidiosa</i> using Nanopore sequencing. (Draft in progress)

Gain International experience on <i>Xylella</i> symptoms, ecology, diagnostic and sampling approaches.	International travel to Brazil, USA and Europe in May and June 2023 to gain experience in the countries that had outbreaks of disease due to <i>Xylella</i> by visiting laboratories and field tour.	International travel plans include visits to <i>Xylella</i> spp. affected regions in Apulia, Italy, Spain, France at the end of May and June 2023. Project participants will meet experts, including Dr Blanca Landa (Institute for Sustainable Agriculture, CSIC, Cordoba), Dr Sophie Cesbron, Dr Marie Agnes-Jaques , Dr Anne-Laure Boutigny and Dr Perrine Portier, and spend time in field and in laboratories to gain experience in working with <i>Xylella</i> spp. cultures and infected plant materials. To gain maximum experience, a visit to Sao Paulo, Barzil, was undertaken in May 2023. Project participants met with Prof. Helvecio (pathologist) and team (Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo), to spend time in field and in laboratories to gain experience in working with <i>Xylella</i> spp. cultures and infected plant materials. DNA of <i>X. fastidiosa</i> spp <i>pauca</i> was obtained. Time was also spent with Prof. Joao Roberto Spotti Lopes (entomologist) and team (University of Sao Paulo) to gain knowledge on vectors specific for <i>Xylella</i> spp. and identification. Afterwards, in the first week of June 2023, a visit to California, USA, is planned to meet Dr Burbank, Dr. Chris Wallis, Dr. Jianchi Chen, Dr. Ray Yokomi, Dr. Rodrigo Krugner and University of Berkely meetings with Prof Steve Lindow, Dr Sandy Purcell, Dr Alex Kahn, Dr Patrick Lee and Prof Alex Purcell. Similarly, time will be spent in field and in laboratories to gain experience in working with <i>Xylella</i> spp. cultures and infected plant materials and to learn about vectors. If additional useful diagnostic methodology is identified, it will be used to update the new NDP. New images of <i>Xylella</i>- spp. infected hosts will be included in the NDP. If a permit for importation is acquired additional preserved positive control material for the testing of the NDP assays will be obtained and brought back to Australia. The final contribution will provide the learnings of this trip at a planned SPHD workshop on the <i>Xylella</i> spp. NDP.
New Collaborations	Submission and approval of new projects with International experts on <i>Xylella</i> .	International travel has ensured that the project partners have built international networks, which will further support Australian and New Zealand diagnosticians in the application of diagnostics tools and new and up to date information about <i>Xylella</i> spp biology to support an outbreak response. Based on the success of this research project NSW and AVR have been accepted to participate in a Euphresco project on the 'Diagnosis of <i>Xylella fastidiosa</i>: detection on dormant plants, important for Mediterranean countries 2202-A-406'. This participation has been accepted and DAFF Biosecurity Innovation Fund (submitted in early 2023) is currently under review, which will assist with funding for this work to further contribute the surveillance capability for <i>Xylella</i> spp. in Australia.

Photos/images/other audio-visual material

Photos below were taken at the “Multi-locus target sequencing (MLST) workshop” and field day activity to demonstrate surveillance with Section 9 of the updated *Xylella* spp. NDP and hands on use of the *Xylella* spp. LAMP and RPA assays at the joint Australian Diagnostic and Surveillance Workshop (ADSW), Canberra, 9-11 May 2023.

The workshop on MLST determination with Nanopore sequencing was provided to 20 registered participants at ADSW 2023.



Figure .1 Demonstration of the Minion instrument and software used for nanopore sequencing for MLST analysis of *Xylella* spp. isolates at the ADSW 2023 MLST workshop.

The field day activity to demonstrate surveillance with Section 9 of the updated *Xylella* spp. NDP and hands on use of the *Xylella* spp. LAMP and RPA assays were provided to 30 registered participants at ADSW 2023.



Figure 2. Dr Pragya Kant demonstrating preparation of plant samples for detection of *Xylella* spp. by LAMP or RPA assays at the ADSW field day activity.



Figure 3. Dr Pragya Kant demonstrating preparation of plant samples for detection of *Xylella* spp. by LAMP or RPA assays at the ADSW 2023 field day activity.



Figure 4. Participants doing DNA extraction from plant samples for detection of *Xylella* spp. by LAMP or RPA assays at the ADSW 2023 field day activity.



Figure 5. Demonstrating the reagents for LAMP and RPA kits and setting up the assays for *Xylella* spp. detection at the

ADSW 2023 field day activity.



Figure 6. Plant homogenates prepared for *Xylella* spp. detection by LAMP or RPA using the Plant Lysis Kit from the samples collected



Figure 7. Dr. Pragma Kant (AVR) and Dr. Lucy Tran Nguyen (PHA) receiving the LAMP assay results, *Xylella* was not detected.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Improving preparedness of the horticulture sector for the threat of <i>Xylella</i> .	Improved, rapid diagnostics for <i>Xylella</i> at the genus, species, subspecies and sequence types level. The NDP is now able to detect both <i>Xylella</i> spp, their sub-species and sequence types. It will support detection and surveillance of a broad range of plant hosts.	New assays that were added to the NDP were tested for specificity and sensitivity. All assays were also validated with negative and positive controls in all the collaborative laboratories. The specificity and sensitivity results were similar for the collaborative laboratories despite of using different kits and machine for conducting qPCRs. Generic endpoint PCR, qPCRs were evaluated for the sensitivity and limit of detection was detected as 2.4×10^{-3} to 2.4×10^{-6} ng/μl, which is highly sensitive. No false positive results were observed while testing these assays on the native flora and spittlebugs.	The NDP assays were either developed as new or adopted from the literature. qPCR and LAMP assay described by Harper et al (2010, erratum 2013) were chosen to be the primary test for both lab and in-field testing. These assays have been widely accepted and during the validation, all collaborative laboratories provided the most consistent results for this assay, therefore have been included in the NDP. Gaps were identified to develop new assays that can not only detect but differentiate <i>Xylella fastidiosa</i> and <i>Xylella taiwanensis</i>. One assay is a generic endpoint PCR whose 650 bp product, when sequenced, can identify the species and subspecies. A generic qPCR and a Duplex qPCR that can detect both <i>Xylella fastidiosa</i> and <i>Xylella taiwanensis</i>, For in-field triage of suspected outbreaks and general monitoring and surveillance, The LAMP of Harper et al (2010, erratum 2013) has been updated using a real-time LAMP machine, has been validated overseas (Harper et al. 2010, erratum 2013) and a commercial recombinase polymerase amplification (RPA) assay from Agdia have been recommended for inclusion in the NDP. Both assays can detect <i>Xylella fastidiosa</i> and <i>Xylella taiwanensis</i>. In-field validation assays results are presented in Milestone 104 Appendix 4, Milestone 105 Appendix 4, Milestone 107 Appendix 3. The lab assays validation results were presented in milestones 104 Appendix 3, milestone 105-107 Appendix 2 and Milestone 108 Appendix 3.
Improving surveillance for the horticulture sector	Clear sampling guidelines for important hosts.	The updated NDP has section for sampling from different host. The requirement of the minimum plant tissue and plant parts have been described. This is adopted from the international standards	International protocols were followed to adopt the sampling procedures for <i>Xylella</i>. The detailed information has been added to the updated NDP and provided in the Milestone 105 Appendix 5. The photos of the <i>Xylella</i> infected grapevine samples received from Dr Lindsey Burbank were shown in Milestone 104 Appendix 1

		such as EPPO and IPPC protocols.	
Confidence of using <i>Xylella</i> NDP and building <i>Xylella</i> diagnostic capability of Australia and New Zealand equivalent to the international diagnostic standards	Validation of laboratory and field tests in an Australian environment	The updated NDP has assays that are robust and accurate. The extensive testing of the native plant samples undertaken in all key laboratories of Australia and New Zealand to rule out any positional cross reactions.	403 plant samples covering 49 genera were tested and no false positives were recorded. The list of the plants has been made and attached to the report for the record. The application of the NDP assays for the surveillance will allow accurate testing of the plant material with fast turnaround. It will provide confidence declaring areas of freedom. It will provide opportunity to further test native plant material and vectors in different seasons and location. These results were presented in Milestone 107 Appendix 4 and Milestone 108 Appendix 1.
A trans-tasman diagnostic capability for <i>Xylella fastidiosa</i> that ensures all major node laboratories are proficient in harmonised diagnostic testing protocols (in the form of a National Diagnostic Protocol) to ensure diagnostic preparedness for an incursion of <i>Xylella fastidiosa</i> .	Harmonisation of testing protocols used across Australia and NZ via the creation of an updated National Diagnostic Protocol (NDP)	All the assays were tested by the four major node laboratories in Australia and the major node laboratory in NZ on same positive and negative DNA panels before adding to the NDP.	The four major node laboratories in Australia and the major node laboratory in NZ established the capability to run key diagnostic tests included in the NDP for the detection and identification of <i>Xylella</i> to species, subspecies and ST taxonomic levels. Establishment of these protocols via SOPs and experience using them is a significant outcome for Australia's and New Zealand's preparedness for a potential incursion or outbreak of <i>Xylella fastidiosa</i> . Results are presented in Milestone 106 Appendix 1, Milestone 107 Appendix 2 and Milestone 108 Appendix 3.
Building capacity of Australasian countries from the threats of <i>Xylella</i>	Ensuring that major node laboratories in Australia and New Zealand have the appropriate resources to test for <i>Xylella fastidiosa</i> (e.g. positive controls) and are proficient in testing protocols for the detection and identification of <i>Xylella fastidiosa</i> listed in the NDP.	The work of this project was carried out in collaboration with the Industry of Primary Industries, New Zealand, which is a major laboratory to deal with the <i>Xylella</i> diagnostics and will support to other laboratories in New Zealand. They have permit to import live cultures and infected plant material which was vital for the development of the updated NDP. They will hold the inventory and can supply DNA to	New Zealand collaborators have got hands-on experience on sampling and culturing <i>Xylella</i> , which would help we want to establish <i>Xylella</i> culturing in Australia laboratories. NZ sourced <i>Xylella fastidiosa</i> strains and infected plant material from international collections and collaborators. Developed a protocol for <i>Xylella fastidiosa</i> isolation from infected plant tissue. Optimized <i>Xylella fastidiosa</i> propagation and long-term storage. Protocols were also developed for purification of high-molecular weight DNA from infected plant leaf petioles and verified <i>Xylella fastidiosa</i> identity. Results were provided in Milestone 104 and 105 Appendix 1,

		Australian laboratories when required.	
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Monitoring and evaluation

Table 3. Key Evaluation Questions

Key evaluation questions	Project performance	Continuous improvement opportunities
Effectiveness		
1. To what extent has the project achieved its expected outcomes? To what extent has the project improved Australia's diagnostic capability and preparedness for the threat of <i>Xylella fastidiosa</i>? To what extent are the Australian diagnostic laboratories proficient in the detection of <i>X. fastidiosa</i>? Have the validated standard operating procedures been included in the NDP in Australia and NZ?	The project fully achieved the expected outcomes. A trans-tasman diagnostic capability for <i>Xylella fastidiosa</i> has been established that ensures all major node laboratories are proficient in harmonised diagnostic testing protocols (in the form of a National Diagnostic Protocol) to ensure diagnostic preparedness for an incursion of <i>Xylella fastidiosa</i>. The updated NDP includes appropriate sampling strategies and the latest and innovative diagnostic tools, technologies, and approaches for the laboratory for rapid and accurate <i>Xylella</i> spp. detection and identification to the subspecies level and in-field detection capability ready for application by field officers, that have been validated for use in Australia. This has ensured that Australia and New Zealand has fit for purpose tools and strategies to identify <i>Xylella</i> spp and to support and outbreak response. The five project partners are the key node laboratories across Australia and New Zealand: all have established and are proficient in the use of the diagnostic tools incorporated into the NDP because of the development and validation processes undertaken throughout the project.	To ensure the key node laboratories remain proficient in the use of the NDP and proficiency testing program to test capability should be established. The NDP should be regularly reviewed: as new technologies become available for <i>Xylella</i> spp. detection, identification, and surveillance, they should be evaluated and, if fit-for-purpose, they should be validated for incorporation into the NDP. Any new technologies should be established in the five key node laboratories. This will ensure Australian <i>Xylella</i> spp. diagnostics are delivered to a world-class standard Ongoing training is required to ensure field officers across all Australian and New Zealand jurisdictions have the capability to apply the sampling strategies and in-field diagnostic technology for <i>Xylella</i> spp. detection.
Relevance		

Key evaluation questions	Project performance	Continuous improvement opportunities
2. How relevant was the project to the needs of intended beneficiaries? To what extent has the project met the needs of industry levy payers? To what extent has the project met the needs of the National Diagnostic Network and Diagnostic Laboratories in Australia and New Zealand?	The project has met the relevant SIP outcomes identified in the logic model for the project (Milestones 102) for each of the industries listed: Apple and Pear, Citrus, Cherry, Dried Grape, Macadamia, Nursery, Olive, Prune, Raspberry and Blackberry, Strawberry, Summer Fruit, and Table Grape. By improving and maintaining Australia's and New Zealand's diagnostic capability for detection, identification, and surveillance for <i>Xylella</i> spp. the industries are safeguarded for biosecurity risks. Rapid and accurate diagnosis mitigates risk and enables rapid response and management to ensure sustainability, productivity, quality and market access. The project has met the needs of the National Diagnostic Network and Diagnostic Laboratories in Australia and New Zealand by addressing Australia's National Plant Biosecurity Diagnostic Strategy to: • Strengthen national and international diagnostic connections • Develop the expertise to enable the delivery of world class plant pest diagnostics • Promote the use of innovative tools, technologies and approaches for improved diagnostics • Ensure appropriate, sustainable and coordinated resourcing to support the diagnostic system.	Training of new staff in key node laboratories and of diagnosticians in other laboratories in the application of the updated NDP will ensure that Australian and New Zealand retain diagnostics capability to support and protect industry from an outbreak of Australia's No. 1 National Priority Plant Pest. Continuous improvement to the NDP, as described above, will ensure Australia and New Zealand have fit for purpose tools and strategies to identify <i>Xylella</i> spp, to meet the relevant SIP outcomes for horticultural industries. It will ensure that both countries have a world-class coordinated plant biosecurity diagnostic approach to <i>Xylella</i> spp. built on expertise shared through national and global collaboration and that they have the highest calibre of plant pest diagnostic expertise to identify <i>Xylella</i> spp
Process appropriateness		
3. How well have intended beneficiaries been engaged in the project? Have regular project updates been	Project updates have been delivered to industry via milestone reports and this final report. Thirteen presentations have been	Further communication to deliver the high-quality outcomes of this project directly to horticultural industry stakeholders could be done in the form of industry articles in relevant

Key evaluation questions	Project performance	Continuous improvement opportunities
provided through industry communication avenues to all stakeholders? To what extent has biosecurity preparedness information been distributed to biosecurity agencies and industry (e.g Plant Health Australia, SPHDS)?	given throughout the project to a range of stakeholders, including diagnosticians, state and federal government, and industry at a variety of workshops, seminars, and conferences (see below under <i>Chapter in a book or paper in conference proceedings</i>).	industry journals and on the Hort Innovation website.
4. To what extent were engagement processes appropriate to the target audience/s of the project? Did the project engage with industry levy payers through preferred learning style? How accessible were the results to stakeholders? Was biosecurity preparedness information delivered in accordance with current requirements of the agencies (e.g. NDP for SPHD)?	The target audience includes diagnostic laboratories, government regulators and industry. The milestone reports and this final report have provided executive summaries or simple summaries as the main communication tool for industry levy payers. For each report these summaries have provided information about: • why was the project undertaken, what is the significance for industry? • what were the project objectives? • what were the key findings/research outputs? • when and how industry will industry benefit from the research outputs? • what are the recommendations emanating from the research? In each report appendices detailing the results of literature reviews and experimental data were provided to ensure stakeholders had access to all relevant information supporting outcomes and achievements. Biosecurity preparedness information was delivered in accordance with current requirements of the agencies through presentations at biosecurity focused workshops, meetings and conferences and this included training	A further workshop is planned for later in 2023, once the NDP is endorsed to provide further awareness of and training in the protocols included the NDP and their application by diagnostics laboratories, field officers and industry.

Key evaluation questions	Project performance	Continuous improvement opportunities
	in relevant technology for diagnosticians and field officers. The updated NDP was submitted to SPHD for endorsement and once it is endorsed it will be made freely available through the National Plant Biosecurity Diagnostic Network website. International travel has ensured that the project partners have built international networks, which will further support Australian and New Zealand diagnosticians in the application of diagnostics tools and new and up to date information about <i>Xylella</i> spp biology to support an outbreak response.	
Efficiency		
5. What efforts did the project make to improve efficiency?	The collaborative nature of this project ensured that efficiencies were made in establishing a world-class coordinated plant biosecurity diagnostic capability for <i>Xylella</i> spp. detection, identification and surveillance across five key node laboratories in Australia and New Zealand to support an outbreak response. Instead of taking months to complete the initial identification to subspecies level, both countries now have access to validated positive controls, specialised media, defined strain typing methods and experience working with the pathogen, to gain a primary detection within and identification to sequence type within days. By establishing diagnostics expertise in multiple laboratories, confirmation of detection can be done quickly so that a rapid and accurate response to a detection can be mounted. This will ensure more samples can be collected and analyses	While New Zealand have a culture collection of <i>Xylella</i> spp isolates, there is no guarantee that the production of positive controls from this resource will always be readily available to Australian laboratories due to the time-consuming nature and expense of growing cultures for production of positive controls. Therefore, it is important to establish this same resource onshore in Australia to ensure Australian laboratories can manage this important resource for Australian laboratories. Importation of <i>Xylella</i> spp. cultures and infected material into Australia is currently not permitted. Provision of an import permit is under review, but has been resisted by some industries who perceive a high level of risk, even though the material will be highly contained. The project team need to work more closely with DAFF and

Key evaluation questions	Project performance	Continuous improvement opportunities
	more efficiently without overwhelming the physical resources of a single diagnostic laboratory. A greater capacity to handle high throughput testing across several laboratories, which use harmonized diagnostics approaches for <i>Xylella</i> spp. detection and surveillance will enable earlier feedback on outcomes from an outbreak. New Zealand have established a collection of <i>Xylella</i> spp. isolates that can be used to provide positive controls to the other key node laboratories and this improved access to positive control material should ensure that all key node laboratories can gain positive controls when required.	industry to inform them of the negligible risk pose by this activity. More efficient, streamlined and harmonized processes are needed to improve access to <i>Xylella</i> spp positive control material and suspect samples within Australia for diagnostic testing or analysis.

Recommendations

Practical application of the project findings:

If an incursion was to happen tomorrow, Australia and New Zealand now have the capability and resources established in key node laboratories to facilitate a rapid diagnosis and response for a broad range of horticultural industries. Instead of taking months to complete the initial identification to subspecies level, both countries now have access to validated positive controls, specialised media, defined strain typing methods and experience working with the pathogen, to gain a primary detection within hours and identification to sequence type within days. This is supported by a verified and validated NDP which will be available to all laboratories wishing to undertake diagnosis once it is endorsed by SPHD in 2023. This NDP should support diagnostics for *Xylella* spp. many years to come. This updated NDP will replace the old NDP which has a primary focus on Pierce's disease of grapevines and uses outdated technology.

This project has delivered significant improvements for *Xylella* diagnostic capacity in Australia and New Zealand. The updated NDP improves upon the EPPO and IPPC protocols.

Once the travel associated with this project is completed there will be:

- Workshops to present the *Xylella* NDP, learning from the overseas travel about symptomatology, diagnostics, surveillance, and management
- The NDP will be updated with any improvements in sampling and culturing procedures and new photos of symptoms.

Possibilities of future RD&E that directly flow from the work undertaken and its results

- It is important to continually review the NDP, especially as new information becomes available about new hosts, vectors and *Xylella* spp, subspecies and sequence types becomes available. It should be updated with new tools as they become available, if they prove to be more effective than the tools that are currently incorporated in the NDP.
- There is a specific need to ensure all laboratories have the diagnostic capability to handle high-throughput testing to support surveillance programs. Specifically, validation of high throughput DNA extraction is required.
- Further validation of plant hosts across different seasons and locations in Australia and New Zealand will improve upon the current validation and provide greater confidence in the NDP (expanding on what we have already done).
- There is a need to improve methodology to distinguish mixed infections of different subspecies and sequence types of *Xylella* spp. :
 - Use pan-genomic comparisons to find Unique Molecular Identifies (UMIs)
 - Develop a rolling circle amplification method which that can be used for tagging individual DNA molecules from different strains (Bi et al 2021; Karst et al 2021; Baloğlu et al 2021).
- Evaluate the methodology (ring testing) and laboratory proficiency to detect *Xylella* in naturally infected plant and insects samples or samples spiked with live cultures– this requires the importation of live cultures. This will ensure laboratories gain experience in handling live specimens which is critical to ensure accurate diagnosis of *Xylella*.
- Further development of a new LAMP assay for *Xylella* spp, including subspecies and sequence types, that is based on a new generic region was identified during the genomic comparison of different *Xylella* isolates (see Milestone 106, table 5). This methodology has greater specificity detection of the different species, subspecies and sequence types which could be helpful for surveillance during an outbreak response. However, the sensitivity needs improvement.
- To better inform surveillance there is a need to undertake transmission experiments:
 - Identify potential local vectors and evaluate their transmission and acquisition efficiency.
 - Identify feeding preferences of vectors occurring overseas to determine their risk to Australian and New Zealand natural environments and crops.
 - Compatibility studies, symptoms expression studies, understanding vector transmissibility and plant- vector interactions.
 - Further evaluation of the sensitivity and specificity of the NDP protocols for detection and identification of

Xylella spp. in local potential insect vectors.

- Identify susceptibility of native plant species to the different *Xylella* spp, subspecies and sequence types.
- Consideration to update contingency plan for *Xylella* with the use of the updated NDP that involves use of HTS and faster turnaround for detection.

Development and adoption activities that would ensure full value from the project's findings for industry

The implementation of the NDP will ensure accurate and fast diagnostics in case of an incursion. The NDP assays have been validated and are in place in all key node diagnostics laboratories (AVR, NSW DPI, NZ PHEL, QDAF and WA DPIRD).

- In addition, to the key node laboratories it will be critical to improve capability in key laboratories of other jurisdictions to ensure their preparedness in case of a *Xylella* spp. detection.
- An inventory and collection of *Xylella* spp. isolates have been established in New Zealand for the vouchered *Xylella* strains which is available to Australia. This is a valuable resource to ensure access the DNA quickly to assist diagnostics.
- The information generated such as list of the native plants which has been tested, characterization of the positive controls and *Xylella* spp. genomes available in NCBI and databases will serve as prime source of information and will be made available to any laboratory for diagnostic purposes, which will enable them to quickly establish capability.
- Training across a broad range of laboratories will ensure the workload can be shared nationally for a rapid response to an outbreak.
- Ensuring laboratories have high through put capability will also be critical to facilitate a response to an outbreak and for proof of freedom testing.

Refereed scientific publications

Manuscripts in preparation:

Manuscript 1: A novel nanopore sequencing-compatible generic PCR assay that can detect and differentiate *Xylella* at the species and subspecies level

Johanna Wong-Bajracharya et al. 2023

- This manuscript focuses on the endpoint assay and its ability to identify both the species and subspecies of *Xylella*. It is anticipated this manuscript will be sent to Hort Innovation for approval to publish in 2023.

Manuscript 2: The testing and evaluation of quantitative PCR assays for the detection of *Xylella* species in the context of Australian biosecurity

- This manuscript focuses on the development of qPCR assays during this project. It is anticipated this manuscript will be sent to Hort Innovation for approval to publish in 2023.

Manuscript 3: High-quality full genome assembly of historic *Xylella fastidiosa* strains from ICMP collection using a hybrid sequencing approach to be submitted as a Microbiology Resource Announcement paper, American society for Microbiology.

- This manuscript is focused on the assembly of the new six ICMP *Xylella* isolates whose genomes are not present in GenBank. We did both Illumina and nanopore sequencing to make high-quality hybrid genome assemblies to be submitted to GenBank. In addition, comparative bioinformatic studies were done on *fastidiosa* and multiplex subspecies using ANI, deciphering differences in the virulence genes and more. Draft to submit the genome announcement is attached (Appendix 7).

Manuscript 4: Identification of sequence typing of *Xylella fastidiosa* using Nanopore sequencing.

- This manuscript describes identification of Sequence Typing based on the MLST amplicons sequencing with Minion and using up-to-date bioinformatics tools designed for nanopore sequencing. There is only one manuscript available in literature by describing it using old bioinformatics methods. It is anticipated this manuscript will be sent to Hort Innovation for approval to publish in 2023

All manuscripts will be submitted to peer-reviewed plant pathology journals.

Chapter in a book or paper in conference proceedings

All presentations are provided as PDF as Appendix 7.

1. Toni Chapman: *Xylella* spp.: The journey to develop a new National Diagnostic Protocol, APPS 2021.
2. Pragya Kant: Improved diagnostics for *Xylella fastidiosa* through high throughput sequencing, APPS 2021.
3. Toni Chapman: An Update on National Diagnostic Protocol *Xylella* spp. PIC@PEQ Seminar series on 29th of April 2021.
4. Rachel Mann: Diagnostic preparedness for the threat posed by *Xylella* in Australia: Infield detection tools, Plant Biosecurity Research Symposium 2022, in Adelaide, in May 2022.
5. Toni Chapman: The journey to develop a new and updated National Diagnostic Protocols – Key learnings from Citrus Canker and *Xylella* spp. NDP's. NDP online webinar on 23rd November 2022.
6. Pragya Kant: Development of the new NDP for *Xylella* spp identification, biannual meeting between DAFF at Victoria (Mickleham) and AVR staff on 18th July 2022.
7. Pragya Kant: Improved diagnostics for *Xylella fastidiosa* through high-throughput sequencing, Annual Diagnostic and Surveillance Workshop held at AgriBio in Victoria on Wednesday 31st September 2022
8. Pragya Kant: Nanopore in diagnostics, workshop on advances of Molecular diagnostics, September ADSW 2022.
9. Rebecca Roach: Bacterial diagnostics: recent advances, 'ologist workshop on 6 the September 2022, Ecosciences Precinct, Dutton Park.

10. Johanna Wong-Bajracharya: How to be generic but specific? A nanopore sequencing-compatible PCR/qPCR assay for detection and differentiation of *Xylella fastidiosa* and *Xylella taiwanensis*, 9-11 May 2023 ADSW, Canberra.
11. Pragya Kant: Multi-locus target sequencing (MLST) workshop, 9-11 May 2023 ADSW, Canberra.
12. Pragya Kant and Lucy Tran-Nguyen: Field activity preparation *Xylella* NDP: LAMP and Agdia assays, 9-11 May 2023 ADSW, Canberra.
13. Pragya Kant: Targeted sequencing approaches for *Xylella* identification and surveillance, 9-11 May 2023 ADSW, Canberra.

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Intellectual property

No project IP or commercialisation to report

Acknowledgements

We acknowledge Dr. Rachel Mann who led the project for first two years before moving to Biosecurity Agriculture Services, Agriculture Victoria. We thank Dr Adrian Dinsdale from DAFF and Dr Piotr Trebicki (former AVR) for providing preserved insect specimens and native spittle bugs for testing with the assays included in the NDP. Thanks to the International collaborators who have agreed to host project teams and spending time to gain experience with *Xyllea* and its diseases.

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Appendices

1. Native Host range tested in Australia and New Zealand- Word document
2. Inventory of *Xylella* strains and information- Excel document
3. Draft National Diagnostic Protocol for detection and identification of *Xylella* spp (**Confidential – not for publishing or distribution**).
4. Overseas travel
5. SOP for MLST using MinION
6. SOP In-field tests
7. Presentations

Appendix 4- International travel to gain experience in diagnosing *Xylella* spp.

The project teams have organised international travel to Brazil, USA and Europe to visit experts in *Xylella* spp. detection, identification and management.

- Dr Pragya Kant (AVR) travelling to Brazil and the USA, 21st May-5th June
 - Dr Helvécio Della Coletta-Filho, Instituto Agronômico de Campinas, Centro de Citricultura Sylvio Moreira, Cordeirópolis, SP, Brazil
 - Prof Joao Lopes, Department of Entomology, University of Sao Paulo, ESALQ/USP, Piracicaba, SP, Brazil,
 - Dr Lindsey Burbank and colleagues at USDA, SJVASC Parlier, California, USA
 - Dr Rodrigo Almeida and colleagues at University of California, Berkeley, USA.
 - At the time of submission of the final report Dr. Kant had completed the first week of her travel and the summary of findings is provided in Appendix 4.
- Dr. Toni Chapman (NSW DPI), Dr. Monica Kehoe (WA DPIRD) and Dr. Luciano Rigano (NZ MPI) travelling to Italy, Spain and France 29th May – 24 June:
 - Dr. Blanca Landa, Institute for Sustainable Agriculture, CSIC, Alameda del Obispo S/N, Córdoba, 14004 Spain (travel in Italy and Spain)
 - Dr Sophie Cesbron and Dr Marie Agnes-Jaques, INRAE, Angers, France
 - Dr Anne-Laure Boutigny, Anses, Plant Health Laboratory, Bacteriology Virology GMO Unit, 7 rue Jean Dixméras, 49044 Angers cedex 01, France
 - Dr Perrine Portier French Collection for Plant Associated Bacteria, Institut Agro, INRAE, Univ Angers, IRHS, SFR QUASAV, CIRM-CFBP, F-49000 Angers, France.

It is anticipated that the travel will lead to an update the bacteriological protocols in the new NDP and provide new images of *Xylella*-infected hosts. Additional preserved positive control material will be obtained to support the development and application of the NDP. Learning about vector movement and containment will inform monitoring and surveillance strategies for *Xylella* spp.

Final travel reports will be submitted to Hort Innovation upon return from each region. A seminar will be delivered within AVR on *Xylella* spp, and the development of the NDP by Dr Pragya Kant. A dedicated *Xylella* workshop is being organised along with APPS 2023, Adelaide later in the year. At this workshop all project partners who travelled overseas will share knowledge and images on the disease, sampling of live and dormant plant material and the information about the updated NDP will be shared. This workshop is aimed at a range of stakeholders including industry, biosecurity policy makers, emergency response teams, and diagnosticians.

Dr Pragya Kant (AVR): Brazil and the USA, 21st May-5th June

Table 1: Itinerary of Dr Pragya Kant travel for Brazil and USA. Shaded area indicates completed activities as of 31st May 2023. At the time of final report submission Dr. Kant had completed her visit to the Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo, Brazil and the University of Sao Paulo, Brazil (Figure 1). Her experience in Brazil is reported below

Date	Country / City	Appointment purpose (meeting, invitation, event etc.)	Appointment participants
May 20 Saturday	Australia- Sao Paulo, Brazil	Travelling Sydney- Auckland- Santiago- Sao Paulo	Dr Pragya Kant
May 21 Sunday	Sao Paulo	Travelling Sao Paulo-Campians- Araras	Dr Pragya Kant
May 23 Monday	Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo.	- Meetings with Dr Helvecio and his team - Discissions on the Xylella and HLB managements and interactions with PhD students and Post Doctorate fellows. Gained information about different projects they are doing on CRISPR editing, transgenic citrus with <i>Bt</i> genes, transformation system. - Saw experiments on feeding and colonisation of psyllids on transgenic citrus plants - Interaction with Post Docs and students	Dr Pragya Kant, Dr. Helvécio Della Coletta-Filho and his team
May 24 Tuesday	Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo	- Media preparation for Xylella - Diagnostic Centre visit - Glasshouse visit for CVC, OQDS symptoms - Photos taken	Dr Helvécio Della Coletta-Filho, Dr Pragya Kant, Dr Alessandra A. De Souza, Ms. Maria Fernanda Verissimo de Oliveira
May 25 Wednesday	University of Sao Paulo, campus to meet Prof. Joao Roberto Spotti Lopes	- Symptoms, sampling, and <i>Xylella</i> isolations - Vectors surveillance and Identification - Visited Glasshouse to see the colonies of sharpshooters - Citrus plants infected with CLas and CLam - Looked the collections of sharpshooters, 27 were specific for Olives and 13 were specific for Citrus.	Dr. Helvécio Della Coletta-Filho, Ms. Maria Fernanda Verissimo de Oliveira, Dr. Pragya Kant Dr. Joyce Adriana Froza and Prof. Joao Roberto Spotti Lopes
May 26 Thursday	University of Sao Paulo, campus to meet Prof. Joao Roberto Spotti Lopes	- Discussions on disease ecology of CVC and HLB. How CVC is managed, and it is not a big problem in Brazil. - Presented seminar on Australian work for updating NDP and diagnostic capability towards Xylella preparedness	Prof. Joao Roberto Spotti Lopes, Pragya Kant, Alessandra, Mariana, Frenanda, Helena, Dr. Paulo Camargo, Mr. Bruno Rampim
May 27 Friday		- Field visit - Meeting the head of Citrus institute Dr Marcus Machado - Writing reports and notes	Dr. Pragya Kant, Dr. Helvecio, Marcus, Frenanda and Helena

Saturday May 27	Araras to Campians to Sao Paulo- flight to Dallas	Travelling to Dallas	Dr. Pragya Kant
Sunday May 28	Dallas to Fresno	Travelling to Fresno	Dr. Pragya Kant
Monday May 29- Wednesday May 31	United States Department of Agriculture (USDA), Parlier, USA	Glasshouse work, Field work and meetings with <i>Xylella</i> project leaders, scientist and students. Presenting a talk on AVR <i>Xylella</i> projects	Dr. Pragya Kant, Dr. Lindsey Burbank, Dr. Rodrigo Krugner, Dr. Ray Yokomi, Dr. Chris Wallis, Dr. Jianchi Chen
Thursday June 1	San Francisco	Travel to San Francisco	Dr. Pragya Kant
Thursday June 2	University of California, Berkely, San Francisco, USA	Meetings with scientist and students at Almeida's lab on ecology, vector surveillance and containment strategies.	Dr. Pragya Kant, Prof. Steven Earl Lindow, Prof. Sandy Purcell, Dr. Alexandra Kahn, Dr. Patrick Lee
June 3- June 5	San Francisco	Travelling San Francisco to Sydney - Melbourne	Dr. Pragya Kant

Visit to the Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo,



Figure 1. Dr. Pragya Kant visited the Citrus Center Sylvio Moreira Cordeirópolis, Araras, Sao Paulo, Brazil to gain experience with *Xylella* spp. in the lab oratory and field. A: The Citrus Center where all laboratories, greenhouses and diagnosistics centre is located. It is a main hub for citrus research in Brazil. B: Pragya with the scientists and students working at the Citrus Center.

Key messages from the visit at Citrus Centre for the control of Citrus Variegated Chlorosis (CVC)

Research from this institute resulted in the recommendation and adoption of the propagation of the citrus plants under net houses to control Citrus Variegated Chlorosis (CVC) disease which is caused by *Xylella fastidiosa* subsp. *pauca*. All citrus mother plants have been maintained in net houses since 2003 and this centre is the sole source for the bud supply to Brazilian nurseries, where they are grafted and sold to the growers. The citrus mother plants are free from seven pests and diseases including CVC, huanglongbing (HLB, *Candidatus Liberibacter* sp.), nematodes, *Phytophthora* spp., blackspot (*Guignardia citricarpa*), citrus tristeza virus (CTV) and citrus canker

(*Xanthomonas citri* subsp. *Citri*). The mother plants are tested annually for these diseases and have reduced their impact to industry.

At the centre they have also developed a system to make citrus tolerant to endemic CTV strains: a less virulent strain of CTV that is maintained in the citrus germplasm systemically and transfer this strain to the bud shoots which are supplied to nurseries.

There are three reasons why Brazil have managed CVC:

1. A law was made in 2003 that all nursey plants and propagation is done in net houses only.
2. Rigorous testing of mother plants- only *Xylella* spp. free material was used for grafting and producing buds.
3. In 2004 HLB was spreading and lots of insecticides were used which were non-selective and controlled vectors of HLB and CVC.
4. At present only 1% CVC is present in citrus growing regions, so not much insecticide control of vectors is done.
5. Micrografting was done for *Xylella* spp free plants.
6. *Xylella* spp doesn't colonise the roots of plants
7. In 2008-2009 the price citrus produce was down, and sugarcane was high, so farmers removed old citrus plants to grow sugarcane and *Xylella* spp inoculum was eradicated.
8. Olive quick decline syndrome (OQDS) is present in Brazil but is confined to the high-altitude area.s Framers are reluctant to control for *Xylella* spp. vectors in this region.

Symptoms in citrus (Figures 2 &3) and olive (Figures 4 & 5) associated with *Xylella fastidiosa* subsp. *pauca*:

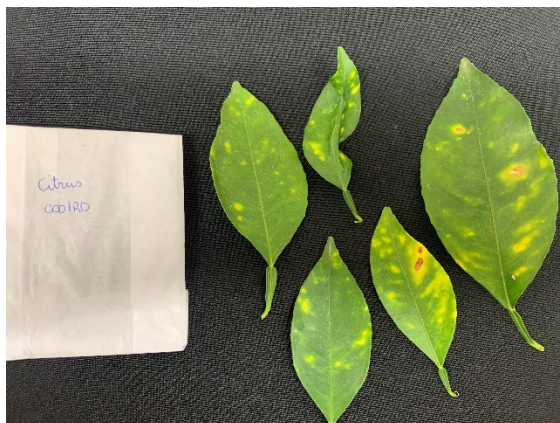


Figure 2. Citrus variegated chlorosis caused by *Xylella fastidiosa* subsp. *pauca* strain common CoDIRO Sequence Type 11



Figure 3. Citrus variegated chlorosis caused by an unknown *Xylella fastidiosa* subsp. *pauca* strain



Figure 4. Olive Quick Decline Syndrome disease caused by *Xylella fastidiosa* subsp. *pauca* strain



Figure 5. Severe symptoms of Olive Quick Decline Syndrome caused by *Xylella fastidiosa* subsp. *pauca*. Note the leaf browning and curling. To sample for *Xylella* spp. the sampling site should be 15-20 cm below the tip, where tissue is alive, as the bacterium is more likely to be accumulating in this region of the shoot.

The diagnostics laboratories at the Citrus Center in Brazil are ISO 17025 accredited and they test for CVC, Huanglongbing (HLB, *Candidatus Liberibacter* sp.), nematodes, *Phytophthora* spp., blackspot (*Guignardia citricarpa*), and citrus canker (*Xanthomonas citri* subsp. *Citri*) but not CTV.

X.fastidiosa subspecies *pauca* is the primary cause of disease so a specific qPCR assay (Oliveira et al 2002) is used to test Citrus, Olive and Coffee. Brazil doesn't follow the EPPO or IPPC protocols: the lab doesn't screen generically for *Xylella* spp. and doesn't use Harper et al (2010, erratum 2013) qPCR assay.

Bacterial Isolation: The media used, and practices are the same as mentioned in the updated Australian NDP developed in the project. Since Australia doesn't have permit to get live cultures, it was valuable that Pragya gained this hands-on experience and will be beneficial for AVR building this capability.

CVC symptoms were not present in the field, but Pragya was able to see HLB symptoms in their field trials.

Severe leaf symptoms and fruit drop and presence of psyllids (*Diaphorina citri*) was observed. Due to heavy use of insecticides, psyllids have become resistant. Dr. Helvecio explained the progression of the first HLB symptoms:

1. Early sign is difference in the colour of the leaves in one branch.
2. Smaller leaves and patchy chlorosis in the leaves in the canopy.
3. Higher stages will see the psyllids mostly confined to the young growth.
4. Leaf and fruit drop and deformation of the fruits.



Figure 6. Field visit for citrus plantation looking at citrus Huanglongbing (HLB) symptoms. Field visit for citrus plantation. A: Pragya looking at HLB in citrus. B: Sweet Orange (*Citrus Sinensis*) orchards.

There are some information booklets on diseases of citrus that Pragya received from the Citrus Center, which will be useful to assist disease diagnosis in the future and management.

Visit to University of Sao Paulo to meet Prof. Joao Roberto Spotti Lopes (Entomologist).

Key messages were:

- Vectors surveillance and Identification: Sticky traps and sweeping netting are used to collect vectors for visual identification.
- 27 vector species were specific for Olives, and the dominant species is *Clastoptera* sp.1 (Figure 7)
- 13 vector species were specific for Citrus (Figure 7)
- Colonies of sharpshooters (*Macugonalia Leucomelas*) are maintained for experimental purposes: colonies are maintained on basil plants (*Vernonia condonsata*), to which this sharpshooter species is well adapted. It multiplies well on basil (Figures 7- 8)
- Pragma viewed observed citrus plants infected with '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter americanus*', this experience will be helpful for visual diagnosis in the future.

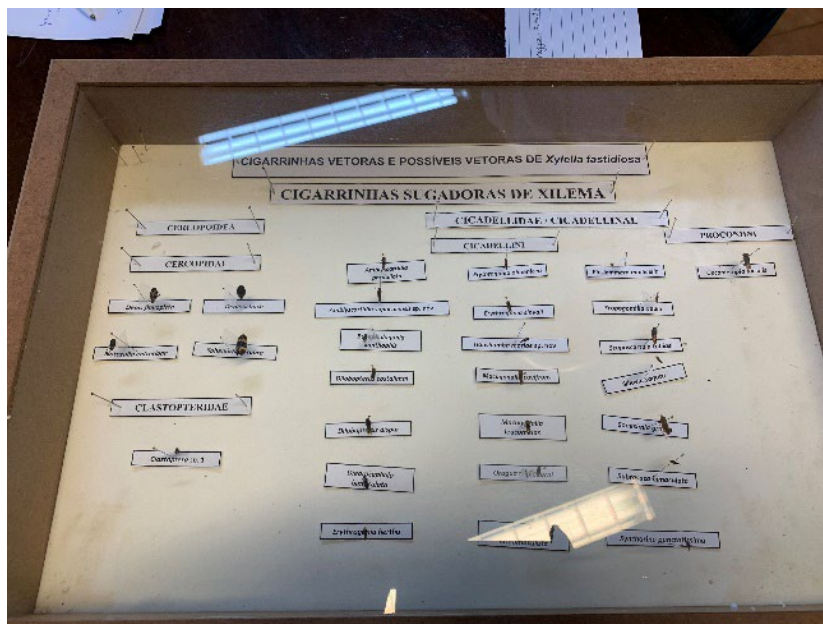


Figure 7. Collection of vectors of *Xylella fastidiosa*.

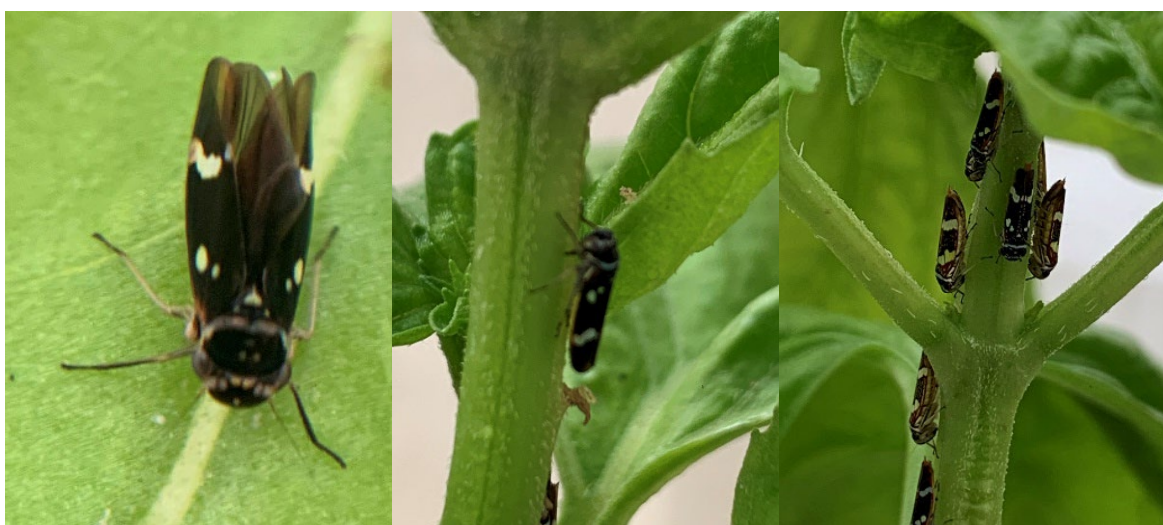


Figure 8. Sharpshooter *Macugonalia Leucomelas* insects on a basil plant.

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Oliveira AC, Vallim MA, Semighini CP, Araújo WL, Goldman GH, Machado MA. 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. *Phytopathology*. 92(10):1048-54.

Dr. Toni Chapman (NSW DPI), Dr. Monica Kehoe (WA DPIRD) and Dr. Luciano Rigano (NZ MPI) travelling to Italy, Spain and France 29th May – 24 June

Table 2: The travel itinerary for Dr Toni Chapman, Dr Monica Kehoe and Dr Luciano Rigano for Europe. They will submit a travel report to Hort Innovation once the travel is completed. The information gained will be used to update the NDP and will facilitate onshore training of the NDP in the future. Toni and Monica are visiting experts from 29 May- 30 June. Luciano will join the trip from 4-24 June

Date	Country/city	Appointment purpose (meeting, invitation, event etc.) and Appointment participants
Monday 29/Tuesday 30 May 2023	Bari	Arrive Italy, meet Dr Blanca Landa
Wednesday 31 May 2023		Visit <i>Xylella fastidiosa</i> subsp. <i>pauc</i> a affected olive groves. Meet with Italian scientists, learn about surveillance particularly with non-symptomatic hosts and management strategies.
Thursday 1 June 2023		
Friday 2 June 2023	Rome	
Saturday 3 June 2023		
Sunday 4 June 2023	Valencia	travel to Valencia
Monday 5 June 2023		Accompany Blanca and her team on trip to visit fields to see multiple difference subspecies of <i>Xylella</i> . Learn how the team conduct surveillance, take samples and observe symptoms.
Tuesday 6 June 2023		
Wednesday 7 June 2023	Majorca	Travel to Majorca with Blanca et al
Thursday 8 June 2023		Accompany Blanca and her team on trip to visit fields to see multiple difference subspecies of <i>Xylella</i> . Learn how the team conduct surveillance, take samples and observe symptoms.
Friday 9 June 2023		
Saturday 10 June 2023		weekend
Sunday 11 June 2023	Seville-Cordoba	travel to Cordoba
Monday 12 June 2023		Work in the laboratory (Cordoba/Blanca), processing surveillance samples, bacterial isolations, DNA extractions, testing methods
Tuesday 13 June 2023		
Wednesday 14 June 2023		
Thursday 15 June 2023	Madrid	travel to Madrid, for flight next day
Friday 16 June 2023	Paris	
Saturday 17 June 2023		
Sunday 18 June 2023	Corsica	Arrive in Corsica - France. Meet Marie Agnes-Jaques and Sophie Cesbron. Participate in annual <i>Xylella</i> surveillance program. Looking at native hosts and collecting samples to process in the lab
Monday 19 June 2023		
Tuesday 20 June 2023		
Wednesday 21 June 2023		
Thursday 22 June 2023	Angers	travel to Angers.
Friday 23 June 2023		Beaucouze, Cedex - France. Work in the INRA laboratory to learn how to isolate <i>Xylella</i> from different host plants
Saturday 24 June 2023		

Sunday 25 June 2023		
Monday 26 June 2023		
Tuesday 27 June 2023		
Wednesday 28 June 2023		
Thursday 29 June 2023	Paris	Begin travel back to Australia
Friday 30 June 2023		Arrive back in Australia

Appendix 5. Standard operating procedure for determining sequence Typing using Nanopore sequencing.

Introduction

Xylella fastidiosa is listed as Australia's number one National Priority Plant Pest (NPPP). There are two species of *Xylella*, *fastidiosa* and *taiwanensis*. *Xylella fastidiosa* has subspecies that can be further classified into sequence type (ST) through multilocus sequence typing (MLST). Due to the genetic diversity of *Xylella fastidiosa* there are a number of different sequence types occurring within each of the different subspecies, with over 80 different ST across the different subspecies. Even within *X. fastidiosa* subsp. *pauca* which has the narrowest host range there are 16 known ST. There is no subspecies and ST related to the *Xylella taiwanensis*.

As part of the Australian National Diagnostic Protocol (NDP) for *Xylella* spp. high-through put sequencing (HTS) tools, including MLST, have been included to detect, identify and characterise the *Xylella* spp. isolates accurately and robustly. Sequencing of multiple taxonomically informative genes or genomes will assist in informing a response and surveillance, since *Xylella* species, subspecies and sequence types can have different host ranges and it supports investigation of the evolutionary relatedness of the isolates. This could be a key for timely management and eradication of this devastating pathogen if it was detected in Australia or New Zealand.

MLST utilises sequencing and analysis of the seven housekeeping genes and based on the variance in each gene an allele number is assigned for that gene. It is officially adopted for the identification of sequence type (ST) for the *Xylella* species and subspecies. The seven house-keeping genes used were *cysG*-600 bp, *holC*- 379 bp, *gltT*- 654 bp, *leuA*-708 bp, *malF*- 730 bp, *nuoL*-557 bp, *petC*- 533 bp.

In this standard operating procedure (SOP) the MLST scheme the identification of sequence types (ST) for *Xf*, which is the current international standard for ST identification as outlined on the EPPO and IPPC diagnostic protocols was adopted. It was combined with nanopore sequencing using a MinION (Oxford Nanopore Technology) to improve sensitivity and turnaround time of MLST analysis directly on plant tissue. This is useful as it bypasses the lengthy time taken to isolate and grow bacteria onto culture plates prior to ST identification. MLST genes products were amplified individually in a PCR, visualised on the agarose gel for the confirmation of amplification and correct size, and sequenced using a MinION device with a ligation kit or a rapid kit according to the manufacturer's instructions.

Standard operating procedures

POLYMERASE CHAIN REACTION

Input DNA can be extracted from the infected plant tissues or the bacterial cultures.

The primer sequences and PCR amplification protocol has been adopted from are from EPPO 2018 with the following conditions using the PCR recipe mentioned in table 1.

95 °C for 2 min, (95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 min) x35*, 72 °C for 5 min

For metagenomic samples- If erratic amplification occurs, the following PCR parameters can be adjusted: dilution of the DNA extract (to limit inhibition) or increase of DNA input, use of a different Taq polymerase/master mix, decrease of annealing temperature from 65°C to 60°C or 58°C or increase of primer concentration from 0.3 to 0.5 µM (recommendation from EPPO 2018). *Note that increasing the cycle number 40 cycles may increase detection of low titre infections in host tissue

Table 1. Typical reagent volumes and concentrations for an MLST PCR reaction for each gene amplification

Reagents	Stock Conc	Final Conc.	Volume
dH ₂ O			15.05 µL
Taq DNA polymerase buffer	X10	1x	2.5 µL
MgCl ₂	50 mM	1.5 mM	0.75 µL
dNTPs	10mM	200 µM	0.5 µL
Forward primer	10 µM	0.2 µM	0.5 µL
Reverse primer	10 µM	0.2 µM	0.5 µL
Taq polymerase	1U/µL	0.2U	0.2 µL
DNA Template			5 µL
Total			25 µL

Table 2. MLST primer list

Primer Name	Primer Sequence 5'-3'	Target Region	Expected product size
leuA-for	GGTGCACGCCAAATCGAATG	2-isopropylmalate synthase	708
leuA-rev	GTATCGTTGTGGCGTACACTG		
petC-for	GCTGCCATTCTGTTGAAGTACCT	ubiquinol cytochrome C oxidoreductase, cytochrome C1 subunit	533
petC-rev	GCACGTCCTCCAATAAGCCT		
malF-for	TTGCTGGTCCTGCGGTGTTG	ABC transporter sugar permease	730
malF-rev	GACAGCAGAAGCACGTCCAGAT		
cysG-for	GCCGAAGCAGTGCTGGAAG	siroheme synthase	600
cysG-rev	GCCATTTTCGATCAGTGCAAAAG		
holC-for	ATGGCACGCGCCGACTTCT	DNA polymerase III holoenzyme, chi subunit	379
holC-rev	ATGTCGTGTTTGTTCATGTGCAGG		
nuoL-for	TAGCGACTTACGGTTACTGGGC	NADH-ubiquinone oxidoreductase, NQO12 subunit	557
nuoL-rev	ACCACCGATCCACAACGCAT		
gltT-for	TCATGATCCAAATCACTCGCTT	glutamate symport protein	654
<i>gltT-rev</i>	ACTGGACGCTGCCTCGTAAACC		

Evaluate amplification gel electrophoresis using 5ul of the PCR reaction loaded into a 2.0% SYBR Safe stained agarose gel (or equivalent) in 0.5x TBE at 100 V for 30-40 minutes. Expected product sizes for each PCR primer set are listed in Table 2.

PCR clean up for library preparation

Pool all the amplicons from one sample and do beads clean up and elute in the 10uM Tris HCl. You can elute in 25 to 40 ul, if there are bright bands in the gel. Quantify all the samples and try to pool in equal amounts into a one tube.

MINION SEQUENCING

Library preparation requirements

1. Ligation or rapid kit can be used and both kits enable barcoding for multiple samples. The Rapid barcoding Kit SQK-RBK004. Both kits have an option to run multiple samples using Barcodes.
2. Flow cell – R9.4.1or R10.
3. Wash kit – EXP-WASH004, if using a used flow cell.
4. Priming kit – EXP-FLP002

The products and protocols are available at <https://store.nanoporetech.com>

The protocol for rapid barcoding kit is https://community.nanoporetech.com/docs/prepare/library_prep_protocols/plasmid-sequencing-using-sqk-rbk004/v/prb_9132_v1_rev03nov2021

The protocol for ligation kit

https://community.nanoporetech.com/docs/prepare/library_prep_protocols/native-barcoding-96-gdna/v/nbe_9121_v109_rev019jan2021

The protocols also required third party items as described in the protocol.

All the seven PCR products were pooled in equimolar concentration for a sample and quantified with qubit. The average DNA needed for the library prep was 100-400 ng.

The sequencing is done using the software for MinION, MinION Release 22.10.7 or above which can be downloaded from <https://community.nanoporetech.com/downloads>

Computer requirements: If it is installed on the GPU, Basecalling and demultiplexing can be done online with MinION software selecting the Fast base calling option or offline using the software Guppy on your desktop or servers.

The base calling and demultiplexing is done using guppy [https://community.nanoporetech.com/downloads#gns\[searchValue\]=guppy](https://community.nanoporetech.com/downloads#gns[searchValue]=guppy)

The amplicon sequencing data requirement is up to 50-100Mb. With the optimum DNA quantity loaded running for 2 hr and up to 0.5 to 1 GB of data is sufficient for multiple samples. The data requirement can be increased or decreased based on the library load and the flow cell health.

MINION SEQUENCING DATA ANALYSIS

MLST protocols have been automated and a script is provided along with the test samples and are freely available on the GitHub page https://github.com/Pragya2019/Xylella_MLST_ONT

The protocols include:

Protocol 1

stringMLST (provide sequence type directly from fastq reads) **CHECK1- what is Sequence Type, makes sense, proceed to confirm with protocol 2**

Protocol 2

Blastn

1. Further confirmation by blasting reads to the MLST gene alleles database.
2. Extracting and filtering reads separately for 7 genes.
3. Visualise the individual gene file to see which allele is in abundance.
4. Output the most abundant reads by Sort, uniq
5. Sequence Type can be confirmed from the *Xylella fastidiosa* profile from PubMLST. **CHECK 2 -if the results match the Protocol 1- confirms Sequence Type.**
https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1
6. The allele reads from the sample fastq are extracted using Seqtk subseq
7. The output from this protocol is used in the Protocol 3.

Blast will analyse individual MLST genes, if analysing all genes together for a sample protocol 3 is used

Protocol 3 To generate consensus of the individual alleles for each sample.

1. **NGSpeciesID** to generate consensus without a reference, it will generate consensus.fasta files for each gene.
2. **Medaka consensus** using the most abundant allele as a reference
3. All the consensus fasta files are concatenated and can be fed into pubMLST website to know the correct allele.

This has a manual step to feed concatenated fasta files to PubMLST website to determine Sequence Typing, if wanted to do automatically on a server, protocol 4 can be used.

Protocol 4

mlst

The consensus fasta files generated for 7 genes are concatenated and sequence type is generated using mlst in command line linux.

REQUIREMENT

The base calling must be done using high- accuracy model.

Nanopore community has software that are frequently upgraded so use the most recent version.

<https://community.nanoporetech.com/downloads>

The base calling and demultiplexing is done using guppy
[https://community.nanoporetech.com/downloads#gns\[searchValue\]=guppy](https://community.nanoporetech.com/downloads#gns[searchValue]=guppy)

If it is installed on the GPU, Basecalling and demultiplexing can be done online with MinION software selecting the Fast base calling option or offline using the software Guppy on your desktop or servers.

Conda or Miniconda needs to be installed before running most of these scripts. Refer to this [link](#) on how to install Conda or Miniconda on your computer. Basic knowledge of bash commands is also required for most of the protocols.

Basecalling

```
/Path_to_software/ont-guppy/bin/guppy_basecaller --version  
/Path_to_software/ont-guppy/bin/guppy_basecaller --device cuda:all:100% --num_callers 8 --config  
/Path_to_software/ont-guppy/data/dna_r9.4.1_450bps_sup.cfg --min_qscore 7 --recursive --input_path  
/path_to_/fast5_pass/ --save_path /Path_to_/basecalled/
```

Demultiplexing

```
/Path_to_software /ont-guppy/bin/guppy_barcode --version  
  
/ Path_to_software /ont-guppy/bin/guppy_barcode --recursive --device cuda:all:100% --barcode_kits EXP-  
NBD104 --num_extra_bases_trim 5 --min_score_barcode_front 70 --records_per_fastq 0 --compress_fastq --  
input_path /Path_to_fast5_pass/guppy_basecalled/pass/ --save_path  
/Path_to_/guppy_basecalled/pass/demultiplexed/
```

PROTOCOL 1

stringMLST <https://github.com/jordanlab>

Installation with conda

```
conda create --name stringMLST  
conda activate stringMLST  
conda install stringMLST
```

to run it requires a database to be build using `--buildDB`
download both alleles and MLST profiles to build it. Make a txt file as given below and run `--buildDB`

[loci]

```
leuA path_to_fasta/leuA.fa  
petC path_to_fasta/petC.fa  
malF path_to_fasta/malF.fa  
cysG path_to_fasta/cysG.fa  
holC path_to_fasta/holC.fa  
nuoL path_to_fasta/nuoL.fa  
glT path_to_fastap/glT.fa
```

[profile]

```
profile Path_to_profile.txt  
  
stringMLST.py --buildDB -c Path_to_file/config.txt -k 35 -P XF
```

or download MLST scheme directly from <https://github.com/jordanlab>

usage

```
stringMLST.py --predict -1 /path_to_fasta_or_fastq -s --prefix /path_to_StringMLSTdatabase -o /sampleST.txt
```

PROTOCOL 2

Make a database of the 7 MLST genes alleles. Download all the allele sequences in fasta files for each gene.

```
makeblastdb -in /path_to_fasta_file -dbtype nucl -title name_db -out name_db
```

MLST allele sequences can be downloaded from pubMLST website

https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadAlleles

scheme	locus	data type	alleles	length varies	standard length	min length	max_length
MLST	leuA	DNA	16	false	708	708	708
MLST	petC	DNA	15	false	533	533	533
MLST	malF	DNA	20	false	730	730	730
MLST	cysG	DNA	36	false	600	600	606
MLST	hoIC	DNA	27	false	379	379	379
MLST	nuoL	DNA	24	true	527 - 557	527	557
MLST	gltT	DNA	18	false	654	654	654

Fastq files changed to fasta

```
seqtk seq -a fastq > fasta
```

```
blastn -db /Path_to_/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -query /path_to_fasta | grep "gene_name_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r > gene_name.txt
```

The above scripts will provide which allele is in abundance for a gene, repeat and grep each gene. Similarly you can blast for rest of the MLST genes e.g petC as mentioned below

```
blastn -db /group/pathogens/plant_micro/projects/Xylella/Pragya/Minion/Xf_dilution/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -query path_to_fasta | grep "petC_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r > gene_name.txt
```

Do similar for all genes and output files for each gene are visualized to know the most abundant allele. The correct allele should be in the abundance. Once all the alleles are known, sequence type is determined from the *Xylella fastidiosa* [MLST profiles](https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1) present on the PubMLST website. https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1.

- **Check 2.** Open the txt files to see the blasted reads are matched to the correct genes, the correct gene allele should be in abundance. From here, all gene allele can be determine and checked with [MLST profiles](#) for *Xylella fastidiosa*. This is a manual step.

Extracting the fastq reads that are corresponding to all the gene such as malF , petC , nuoL, leuA, holC, gltT and cysG.

This output from this step will be needed to run Protocol 3.

PROTOCOL 3

To make consensus for each MLST gene together using NGSpeciesID or Medaka

Subseq these from the sample fastq file. The fastq containing reads for each genes that will be used to make consensus

Subset the allele match reads from the protocol 2 and extract reads for all seven genes.

```
seqtk subseq $originalfile.fastq $one_mlst_gene.txt > $one_mlst_gene.fastq
```

Making consensus of the best match alleles for all seven genes using NGSpeciesID (Sahlin et al 2021) which works by clustering highly similar sequences and makes groups. This method has also been proposed for making consensus for amplicon generated from endpoint PCR assay. Since Nanopore reads has up to 5 % error rates, making consensus of alleles that differ only in few base sequences could create noise while making consensus so if the sequencing is done with latest chemistry 14 kit and using high accuracy model for base calling and demultiplexing that allowed less than 1% error rates NGSpeciesID is good to use.

This workflow has been tested to work on Linux environment with conda installed and it is dependent on the following tools:

[NGSpeciesID](#) It doesn't require any reference to make consensus.

1. Installation with conda

Creating NGSpeciesID environment

```
conda create -n NGSpeciesID python=3.6 pip
```

```
conda activate NGSpeciesID
```

```
conda install --yes -c conda-forge -c bioconda medaka==0.11.5 openblas==0.3.3 spoa racon minimap2 blast
```

```
pip install NGSpeciesID
```

2. Creating medaka environment

```
conda create -n medaka -c conda-forge -c bioconda medaka blast
```

```
NGSpeciesID --ont --fastq $one_mlst_gene.fastq --outfolder $one_mlst_gene_NGSouput --consensus --medaka
```

```
cat consensus_reference_*.fasta > one_gene.fasta
```

Here you should have consensus for each gene for a sample, now concatenate them into one file

When using medaka, the most abundant allele for a gene found in Blastn from Protocol 2 can be used as a reference.

```
medaka_consensus -i ${BASECALLS} -d ${DRAFT} -o ${OUTDIR} -t ${NPROC} -m r941_min_high_g303
```

concatenate all seven genes consensus to a fasta file

```
cat $one_to_sevenMLST_genes_fasta > $sample.fasta
```

3. Determining the sequence type

The Sequence typing is determined by two ways

1. PubMLST – copy and pasting the concatenated all MLST gene “sample.fasta” to determine the profile.
This is a manual step
2. Using command line mlst <https://github.com/tseemann/mlst>

Installation using [Conda](#)

```
% conda install -c conda-forge -c bioconda -c defaults mlst
```

usage

```
mlst $sample.fasta > ST.txt
```

mlst is primarily used on the whole genome sequencing. Assembled contigs are fed into directly, can be ask for specific organism such as *Xylella fastidiosa*

Multilocus Sequence Typing (MLST) of *X. fastidiosa* using Nanopore sequencing

The amplicon of MLST genes are amplified and confirmed by visualization by agarose gel electrophoresis as described above (Table 16a to c) and then sequenced either on Flongle or MinION device. Here we have described the method using MinION.

1. All seven PCR products for each sample are pooled, purified, and quantified using a NanoDrop spectrophotometer and Qubit fluorometers. The average DNA needed for the library prep was 100-400 ng depending on the kit used for the library preparation.
2. Library preparation is done using either Ligation or rapid kit, both kits enable barcoding for multiple samples. The products and protocols are available at <https://store.nanoporetech.com>. The protocols also required third-party items as described in the protocols. The protocol described here is using Ligation kit SQK-109, and flowcell R9.4.1 but new chemistry is available to use Kit 14 and R10 flowcells for sequencing.
3. The sequencing is done using the software for MinION, MinION Release 22.10.7 or above which can be downloaded from <https://community.nanoporetech.com/downloads>
4. The data requirement is up to 50-100 Mbp. With the optimum DNA quantity loaded on the device, sequencing run for 2 hr of the run is sufficient. The data requirement can be increased or decreased based on the library load, flow cell health and kit used for the library preparation.
5. Perform base-calling and barcode removal on the Fast5 files generated after sequencing. This step can be performed in real-time or after execution on the MinKNOW platform or using the command line-based tool ‘guppy’ (Version 6.1.) using the high accuracy mode [https://community.nanoporetech.com/downloads#gns\[searchValue\]=guppy](https://community.nanoporetech.com/downloads#gns[searchValue]=guppy) to get fastq files.

The generated fastq files are analyzed using command line-based tools in a Linux environment. Conda or Miniconda needs to be installed before running most of these scripts. Refer to this [link](#) on how to install Conda or Miniconda on your computer. Basic knowledge of bash commands is also required for the protocols.

The detailed setup, dependencies, and usage of relevant analysis tools could be found on the following GitHub page: https://github.com/Pragya2019/Xylella_MLST_ONT. It also has an automated script to run analysis.

Two protocols are used to confirm results.

PROTOCOL 1

StringMLST <https://github.com/jordanlab> is an assembly- and alignment-free, lightweight, platform-independent program capable of rapidly typing bacterial isolates directly from raw sequence reads. It works well with low read counts as well. It is the fastest and easiest tool to determine ST if the reads are accurate. Therefore, the requirement is to use the high accuracy model for base calling and demultiplexing. It will require installation with Conda.

Installation with conda

```
conda create --name stringMLST
conda activate stringMLST
conda install stringMLST
```

to run it requires a database to be built using `--buildDB`
download both alleles and MLST profiles to build it. Make a txt file as given below and run `--buildDB`

[loci]

leuA path_to_fasta/leuA.fa

petC path_to_fasta/petC.fa

malF path_to_fasta/malF.fa

cysG path_to_fasta/cysG.fa

holC path_to_fasta/holC.fa

nuoL path_to_fasta/nuoL.fa

glT path_to_fastap/glT.fa

[profile]

profile Path_to_profile.txt

stringMLST.py --buildDB -c Path_to_file/config.txt -k 35 -P NM

or download the MLST scheme directly from <https://github.com/jordanlab>

usage

stringMLST.py --predict -1 /path_to_fasta_or_fastq -s --prefix /path_to_StringMLSTdatabase -o /sampleST.txt

PROTOCOL 2

BLASTn- This method utilized two steps, first to make a database of MLST genes downloaded from https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadAlleles using the script

```
makeblastdb -in /path_to_fasta_file -dbtype nucl -title name_db -out name_db
```

secondly, convert the fastq file to fasta

```
seqkit fq2fa reads.fq.gz -o reads.fa.gz
```

and do blast with -max_hsps 1,

```
blastn -db /Path_to_/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -query /path_to_fasta | grep "malF_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r > malF_blast.txt
```

sort and count the occurrence of malF. Do similar for all genes and output files for each gene are visualized to know the most abundant allele. The correct allele should be in the abundance. Once all the alleles are known, sequence type is determined from the *Xylella fastidiosa* [MLST profiles](https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1) present on the PubMLST website. https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1

APPENDIX 6: Draft SOP for detection of *Xylella* using in-field LAMP or RPA testing

Sampling for LAMP or RPA

Plant

X. fastidiosa is confined to the xylem tissue of its hosts. The petiole and the midrib recovered from leaf samples are therefore the best sources for diagnosis, as they contain a greater number of xylem vessels (Hopkins, 1981). It is recommended that in-field testing is conducted on symptomatic plants. Pooled petiole and midrib tissue of plants with larger leaves or for plants with smaller leaves, whole leaves can be used.

Insect

DNA may be extracted from a single insect head or a pool of five heads (Bextine et al., 2004; EPPO, 2018). Only the heads of insects are used because they contain the foregut and mouthparts where *X. fastidiosa* resides (Bextine et al., 2004). For DNA extraction from insects with big heads (e.g. *Cicadella viridis*, *Cicada orni*), only a single head should be used. For small insects (e.g. spittlebugs) whole insects can be used.

DNA extraction for LAMP or RPA

Extraction from plant hosts using Plant Lysis kit (Optigene)

The Plant Lysis Kit is recommended for use for plant DNA extraction based on comparative extraction method results presented in Milestone 105.

Materials, equipment, kits and reagents required

- The Plant Lysis kit (Optigene) includes the following components:
 - Bijoux with ball bearing
 - Lysis buffer
 - Transfer loops (10 µl)
 - Dilution buffer
- Pipette (100-1000 µL)
- Filter tips (1000 µL)
- Scalpel blades
- Cleaning – 80% ethanol



Figure 1. Plant Lysis Kit components

Procedure

1. Transfer 1 mL of lysis buffer to the red capped bijoux. *Note: this can be done before heading into to the field.*
2. Add plant tissue to the lysis tube.
3. Close lid firmly and shake tube by hand for 1 min.
4. Use the 10 μ L loop to transfer homogenised tissue extract from the bijoux to the tube labelled 'dilution buffer' and mix loop into the buffer.
5. The sample in dilution buffer serves as template for the LAMP reaction.

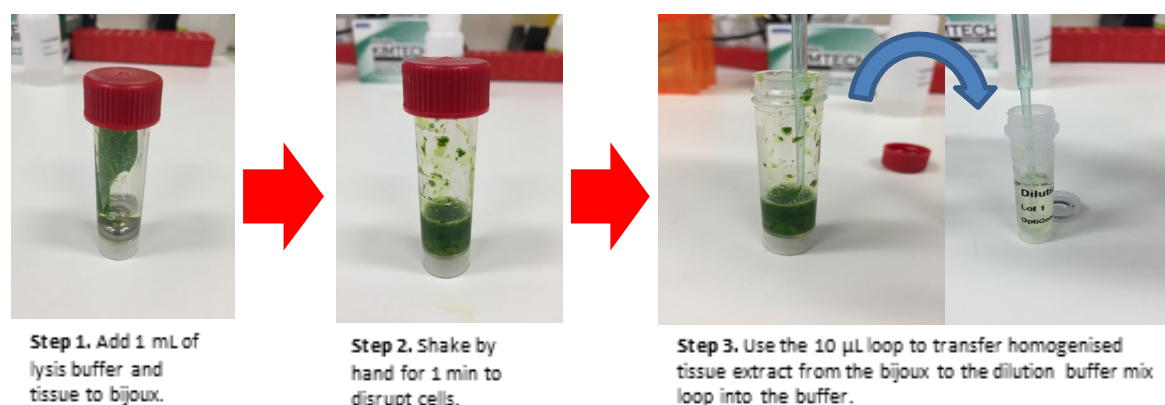


Figure 2. Steps of DNA extraction using the Plant Lysis Kit

Extraction from insect hosts using QuickExtract DNA

QuickExtract DNA solution (Lucigen) is recommended for insect DNA extraction for use with LAMP based on comparative extraction method results and validation across a range of insect genera generated by AVR Projects and publications. These include:

- AJIF Horticulture Development Plan (HDP) CMI 106199
- Phylloxera LAMP (AVR project CMI 106128)
- Brown Marmorated Stink Bug LAMP (AVR project CMI 106136)
- Khapra Beetle LAMP (AVR project CMI 106136)
- Varroa mites LAMP (AVR project CMI 106136)
- Blacket et al. 2020

Materials, equipment, kits and reagents required

- Pipette (20-200 μ L)
- Filter tips (200 μ L)
- 0.2 mL LAMP strip tubes
- QuickExtract DNA solution
- Scalpel blades

- Cleaning – 80% ethanol
- Genie III machine or heat block

Procedure

1. Transfer 100 μ L of QuickExtract solution into 0.2 mL LAMP strip tubes *Note: this can be done prior to going out into the field.*
2. Sever the heads of insects using a scalpel blade and add 1-5 heads to the QuickExtract DNA solution using forceps and close the lid. *Note: the number is dependent on the size of the insect. For small insects (<7mm) the whole specimen can be added to the mix.*
3. Transfer the strip tube to the Genie III or heat block and run it at 65 °C for 6 min followed by 98 °C for 2 min (Total run time = 8 min).
4. The resulting solution can now be used directly as DNA template in LAMP tests.

Draft SOP for in-field detection of *Xylella* spp. using LAMP

The LAMP assay for detection of *Xylella* spp. is an isothermal DNA amplification and detection system. Detection of amplified DNA is measured in real-time detection using a portable fluorometer (Genie III).

Materials, equipment, kits and reagents required

- Filter tips (10 µL, 20 µL, 200 µL and 1000 µL)
- Pipettes (0.5-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL and 8-channel 10 µL multichannel)
- Genie III machine
- 0.2 mL LAMP strip tubes
- Isothermal Mix (In OptiGene kit)
- Sterile molecular grade water
- Gloves
- 1.5 mL Eppendorf tubes
- Positive control
- Sample DNA (from extractions)
- PCR grade water
- Xf LAMP primer mix (preparation instructions below)
- COX LAMP primer mix (preparation instructions below)

Procedure

Please note that completing the following prior to going into the field will simplify the in-field test process:

- Prepare primer mixes (this can be done at any time and stored at -20 °C)
- Prepare LAMP master mixes and aliquot into test strips – must be done on the day and reagents need to be transported on ice in an esky/chilly bin or alternative portable cooling system until required.

Internal amplification control

It is recommended that a LAMP test targeting a universal housekeeping gene within the host (where practical and available) is run on all samples ensure the DNA extraction has been successful and no inhibitors from the insect or plant host tissues (e.g. lignins, polyphenols, pigments, fats, proteins) prevent DNA amplification during the LAMP reaction.

The Cox LAMP test is designed to amplify plant cytochrome oxidase gene sequence (Tomlinson 2010). The gene is highly conserved among plants and the amplification of the Cox gene serves as an internal/extraction control for use with plant samples.

Currently there are no published universal LAMP housekeeping tests for amplification of insect DNA. AVR have a universal LAMP housekeeping test in development but it is not available for inclusion in this report. A bacterial housekeeping gene could also be used here and will be investigated.

Failure to produce amplification with the housekeeper primers may suggest the following:

- Extraction has failed and re-extraction is required; or,
- Inhibitor concentration in sample is too high and is interfering with the activity of the enzyme.
Dilution of the extraction may resolve the issue; or,
- The concentration of the DNA in the extraction is too low and re-extraction is required

Primer mix preparation

Table 2. Primer concentrations for internal amplification control and *Xylella fastidiosa*-specific LAMP assay.

LAMP test for Cox	Primer	Stock concentration (μM)	Amount to add for primer mix (uL)	Conc. in primer mix (uM)	Final conc. In reaction (uM)
Ratios			1:10:5		
Cox housekeeping LAMP (Tomlinson et al. 2012)*	B3	10 μM	10	1	0.2 μM
	F3	10 μM	10	1	0.2 μM
	BIP	100 μM	10	10	2 μM
	FIP	100 μM	10	10	2 μM
	LF	100 μM	5	5	1 μM
	BF	100 μM	5	5	1 μM
Sub total			50		
H2O			50		
Total			100		

* For plant tissues only

Table 14: Primer concentrations for and *Xylella fastidiosa*-specific LAMP assay.

LAMP test for <i>X. fastidiosa</i>	Primer	Stock concentration	Amount to add for primer mix (uL)	Conc. in primer mix (uM)	Final conc. In reaction (uM)
<u>Ratios</u>	-	-	<u>1:8:4</u>	-	-
<u>X. fastidiosa-specific LAMP (Harper et al. 2010, erratum 2013)</u>	<u>B3</u>	<u>10 µM</u>	<u>10</u>	<u>1</u>	<u>0.2 µM</u>
	<u>F3</u>	<u>10 µM</u>	<u>10</u>	<u>1</u>	<u>0.2 µM</u>
	<u>BIP</u>	<u>100 µM</u>	<u>8</u>	<u>8</u>	<u>1.6 µM</u>
	<u>FIP</u>	<u>100 µM</u>	<u>8</u>	<u>8</u>	<u>1.6 µM</u>
	<u>LF</u>	<u>100 µM</u>	<u>4</u>	<u>4</u>	<u>0.8 µM</u>
	<u>BF</u>	<u>100 µM</u>	<u>4</u>	<u>4</u>	<u>0.8 µM</u>
<u>Sub total</u>	-	-	<u>44</u>	-	-
<u>H₂O</u>	-	-	<u>56</u>	-	-
<u>Total</u>	-	-	<u>100</u>	-	-

Specific LAMP detection of *X. fastidiosa*

Real-time LAMP detection of *X. fastidiosa* is performed using the primers designed by Harper et al. (2010 erratum 2013) that target the *rimM* gene. These primers are designed to be species-specific for *X. fastidiosa*. The LAMP reaction details are outlined in Table 3. The template can be used 2-5 ul and adjusting the volume of the water.

A positive control and a negative amplification control should be included in the testing.

A housekeeping LAMP must be run in parallel on plant host extracts to ensure extracted DNA can be amplified.

Table 3. Reaction set up for the Real Time Lamp assays used to detect *Xylella fastidiosa*

Component	Per reaction (25 µL)	X number of reactions
Sterile water	3 µL	
Isothermal Mix	15 µL	
LAMP Primer Mix	5 µL	
Template (extraction)	2 µL	
Reaction total	25 µL	

Note: when calculating for master mix add a minimum of three reactions to the number of samples (positive and negative controls, spare)

5. Make master mix and mix thoroughly by slowly pipetting 10 times.
6. Aliquot 15 µL of master mix into each tube of the LAMP strip tubes.
7. Add 3 µL of sterile water to the negative control tube and close the lid.
8. Add 2 µL of template (DNA extraction) into the reaction tubes and close the lids.
9. Add 2 µL of the positive control DNA into the positive control tube and close the lid.
10. Load the LAMP strip tube into the Genie III and run the below protocol, this protocol can also be saved as a template in the machine.

LAMP Protocol:

65 °C 30 mins, annealing curve 98 °C to 80 °C (rate 0.05 °C/sec), SYBR Green channel

Result interpretation

If target DNA is present, dsDNA will be amplified and an increase in fluorescence from the dsDNA intercalating dye will be visualised on the Genie III. A positive amplification plot will show a sigmoid alcurve reflecting the increase in fluorescence detected (Figure 3). A negative result should stay relatively flat.

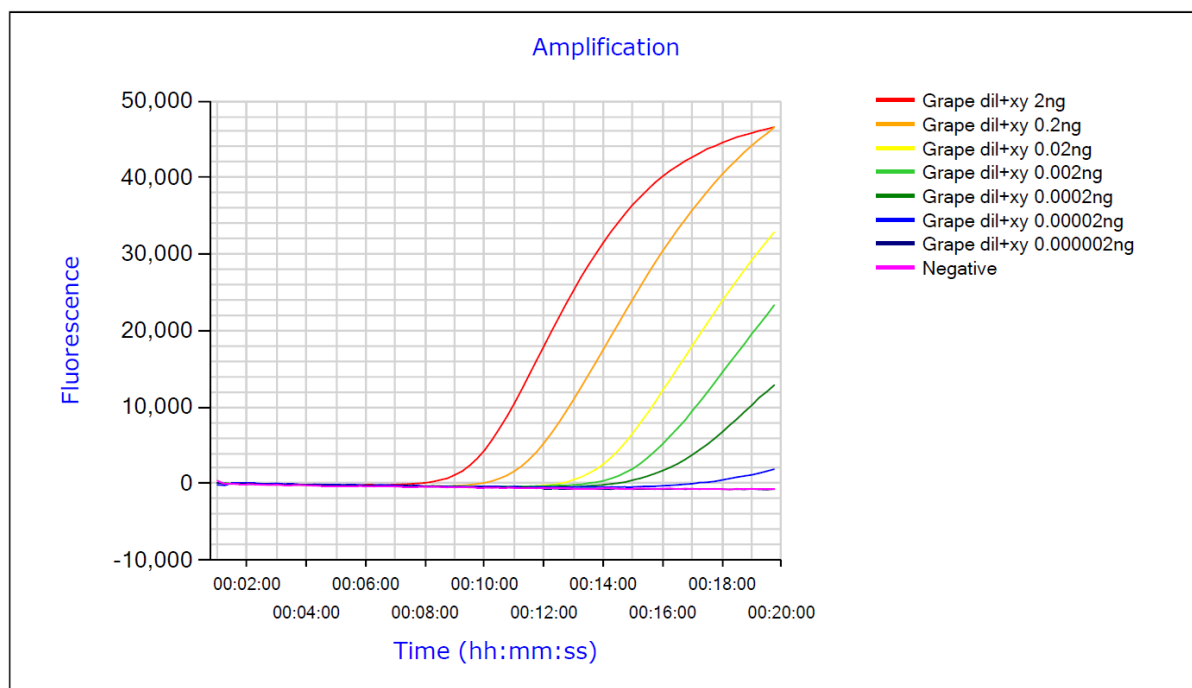
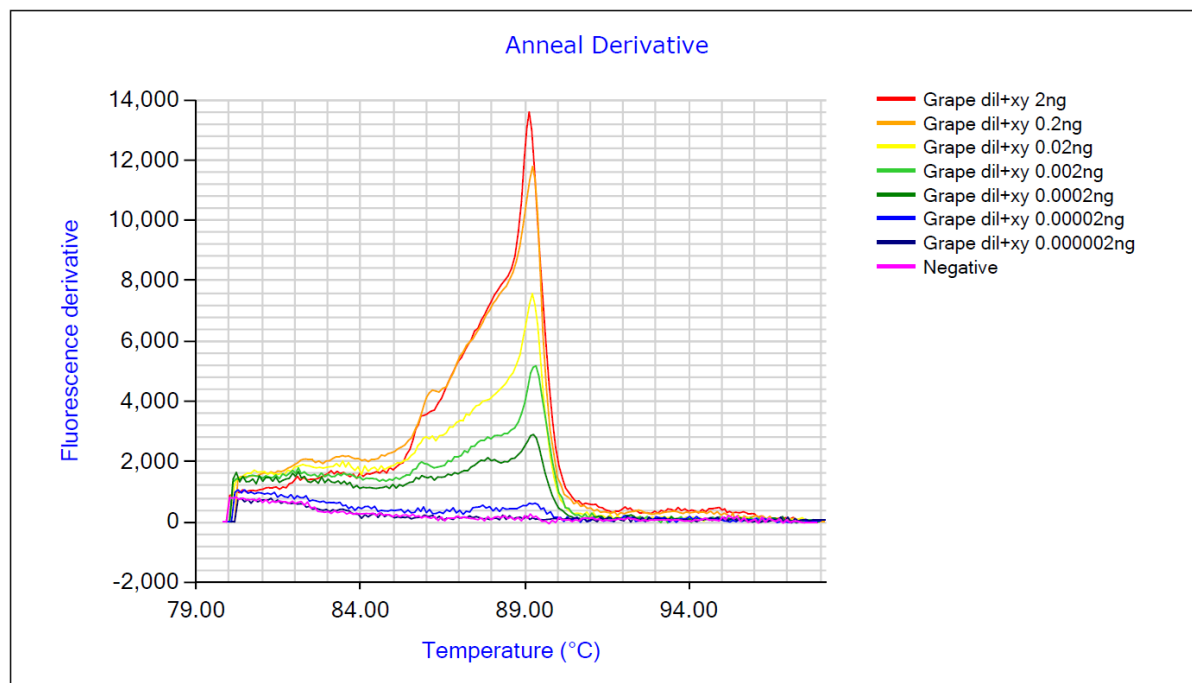


Figure 3. LAMP amplification rate data for six positive (sigmoid) amplification curves (red, orange, yellow, green, dark green and blue) and two negative amplifications (flat) (navy and pink)

As confirmation of amplification of the correct target an anneal step is performed after the isothermal step. Each target will have its own signature anneal temperature which is related to the GC content of the generated amplicon (figure 4). Positive amplification followed by an anneal temperature at the expected range is confirmation of a true positive in a sample.



Well Number	Well Name(°C)	Peak Value(°C)
1	Grape dil+xy 2ng	89.1
2	Grape dil+xy 0.2ng	89.2
3	Grape dil+xy 0.02ng	89.2
4	Grape dil+xy 0.002ng	89.3
5	Grape dil+xy 0.0002ng	
6	Grape dil+xy 0.00002ng	
7	Grape dil+xy 0.000002ng	
8	Negative	

Figure 4. Anneal Derivative data of the amplification products generated in the LAMP reaction in Figure 3. There is a consistent annealing temperature peak value indicating that the nucleic acid composition of the products is the same, confirming a positive result.

The real-time LAMP test will be considered valid only if the following criteria are met:

- the positive control produces a sigmoidal amplification curve with the *X. fastidiosa*-specific primers
- the negative amplification control does not produce an amplification curve with the *X. fastidiosa*-specific primers.
- the extracted DNA template produces a sigmoidal amplification curve with the Cox housekeeping LAMP primers for plant extracts.
- Positive samples have the same annealing temperature as the positive control.

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- PM 7/24 (3) *Xylella fastidiosa*. EPPO Bulletin, 2018. 48 (2): p. 175-218.
- Tomlinson, J. A., Dickinson, M. J., & Boonham, N. (2010). Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology*, 100(2), 143-149.

Draft SOP for in-field detection of *Xylella* using the Agdia Amplify RP® XRT kit

Introduction

The Agdia Amplify RP® XRT kit for *Xylella fastidiosa* is an isothermal DNA amplification and detection system based on Recombinase Polymerase Amplification (RPA). Detection of amplified DNA can be measured in two different ways depending on the resources available; real-time detection using a portable fluorometer; or at the end of the test (end-point detection) using lateral flow devices. The reaction kit, fluorometer and amplicon detection chambers (which includes the lateral flow strips) are commercially available from www.agdia.com:

1. Part Number XCS 34501/0048 (contains reaction pellets in tubes).
2. ADC 98800/0001 AmplifyRP Amplicon Detection Chambers for endpoint detection.

For real-time detection any portable fluorometer could be used, however, Agdia have a device called an AmpliFire® Isothermal Fluorometer (AFR 60400) that is compatible with the tubes from the reaction kit.

Local distributor

Email Susan.Archer@dpiw.tas.gov.au

Department of Primary Industries, Parks, Water & Environment

BIOSECURITY TASMANIA

New Town Laboratories 13 St John's Avenue New Town, Tasmania, 7008

Ph. (03) 61653243

Web www.dpiw.tas.gov.au

Materials, equipment, consumables and reagents

Details of all equipment, instrumentation, consumables and reagents required for the SOP.

- Biosecurity waste bag/biohazard bags (waste disposal)
- Gloves
- 0.2 mL LAMP strip tubes
- Ethanol (70%)
- Scalpel blades
- Forceps
- Weighing machine (optional)
- 1000 µL filter tips
- 200 µL filter tips

- 20 µL filter tips
- Pipette Adjustable single channel 2 - 20 µL
- Pipette Adjustable single channel 20 - 200 µL
- Pipette Adjustable single channel 200 - 1000 µL
- 1.7 mL Eppendorf tubes
- Sterilized molecular grade water
- XCS 34501/0048 kit
- AFR 60400 -AmpliFire® OR Genie III LAMP
- ADC 98800/0001 AmplifyRP Amplicon Detection Chambers

XCS 34501/0048 kit contains following items

- XRT+ reaction pellets for Xf (48 individual tubes)
- PD1 Pellet diluent (55 mL)
- Amp1 extraction buffer (3 x 55 mL)
- Micro centrifuge tubes (50)
- Mesh extraction bags (50)

All kit components should be stored refrigerated (2 - 8 °C).

Before use, allow all kit components to warm to room temperature (18 - 30 °C) for 20 to 30 minutes.

Sample Preparation

X. fastidiosa is confined to the xylem tissue of its hosts. The petiole and the midribs are therefore the best sources for diagnosis, as they contain a greater number of xylem vessels (Hopkins, 1981). Bacteria is most often not evenly distributed through-out an infected plant vascular system. For symptomatic plants it is recommended to sample tissue close to symptomatic tissue (but not necrotic tissue) from multiple parts of the plant for in-field testing. For asymptomatic plants it is recommended that tissue is sampled from multiple parts of the plant

DNA Extraction

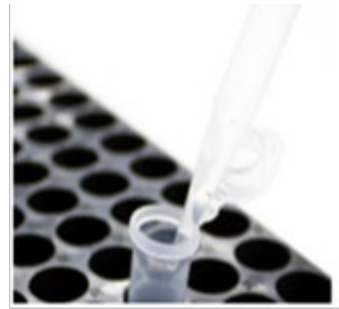
Select 0.3 g or 4-5 petioles, 5-7 cm long of suspect tissue and insert the sample between the mesh linings of the sample extraction bag. Add 3.0 mL of AMP1 extraction buffer to the tissue inside the bag. Extract the tissue by thoroughly macerating it with a blunt object such as a pen. Take 1 mL of PD1 diluent in 1.5 mL tube and add 10 µl of the sample extract for the testing. For maximum sensitivity, allow the extract to incubate for 30 minutes at room temperature before testing. *Note: This test was optimized using a 1:10 tissue to buffer ratio for sample extraction.



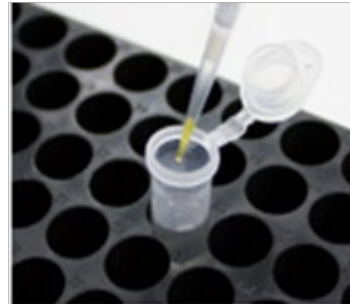
Step 1. Cut petioles from the symptomatic leaves



Step 2. 0.3 gm of petioles cuttings from different parts of the plants



Step 3. Pipette 1mL of PD1 (pellet Diluent) provided with the kit into 1.5mL microcentrifuge tube for each sample.



Step 4. Transfer 10µL of sample extract into the microcentrifuge tube containing PD1 diluent and mix well.



Samples are now ready to be tested.

Figure 1. Steps involved in DNA extraction from plant hosts for detection of *Xylella* using the Agdia Amplify RP® XRT kit

Detection

The detection can be conducted in two ways depending on the equipment available.

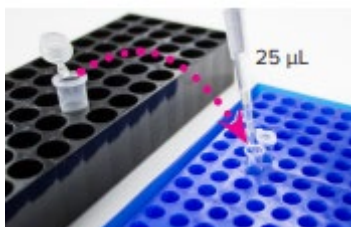
1. Real-time detection using
 - a. AmpliFire® provided from Agdia; or
 - b. Genie III LAMP machine
2. End-point detection using lateral flow chambers provided from Agdia

1a. Real-time detection using AmpliFire

1. Press the “Execute Reaction” button on the AmpliFire®. Then press “Scan Product Code”.
2. (Optional) Scan the barcode found by following the hyperlink on page 1. The barcode scanner is located on the left side of the AmpliFire. Once the AmpliFire has accepted the scan and displayed run method, click “Next”.
3. Follow on-screen prompts to name your reaction and individual sample IDs
4. Remove a canister of reaction pellets from the white foil pouch labelled with the barcode. Then remove a strip of reaction pellets from the desiccated container. While securing the strip of pellets in a 200 µL PCR tube rack, cut the number of reaction pellets from the strip that are intended for use.

Note: Reaction Pellets are light sensitive. Immediately place remaining reaction pellets back into the desiccated tube and then insert the desiccant tube into the foil pouch to protect from light.

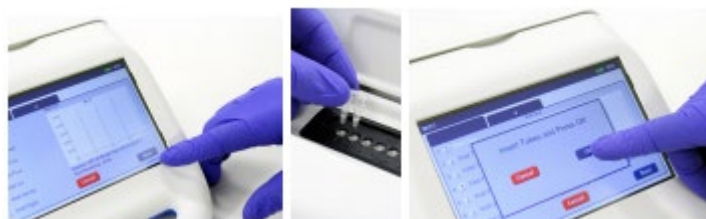
5. Transfer 25 µL from the microcentrifuge tube containing your diluted sample extract into the reaction pellet (clear tube).



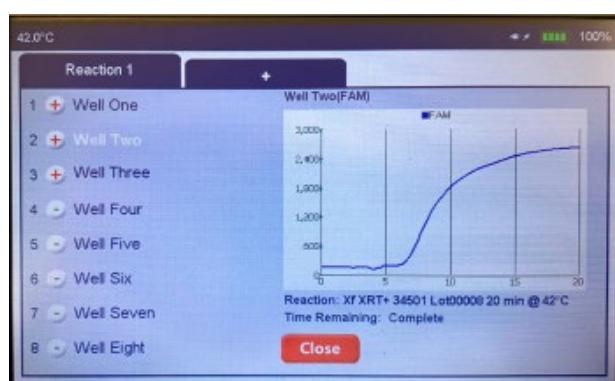
6. Tightly recap the reaction tube. Mix well and centrifuge. If you cannot vortex the reaction, mix by gently flicking the side of the tube. If you do not have a centrifuge available, you may manually shake the liquid to the bottom of the reaction tube

Important: do not transfer more than the prescribed 25 µL during this step. Immediately proceed to the next step once the reaction has been rehydrated.

- Press “Start” on the AmpliFire. Immediately follow the prompts to add your reactions, press “OK”, and put the lid down.



- After 4 minutes of incubation remove the reaction(s) from the AmpliFire. Quickly mix, spin, and reinsert the reaction(s) into the AmpliFire to continue monitoring results. Take care to ensure the tubes are in their original positions and orientations.
- After 20 minutes of total run time the instrument will beep, indicating the test is complete. The test results will be visible next to the well designation on the screen, and should be interpreted as follows:
- (+) = Positive for Xf (-) = Xf not detected (!) = Invalid



1b. Detection in Genie III LAMP machine

- Take out reaction pellets as described above in step 4 based on the samples to be tested.
- Follow steps 5 and 6.
- To run samples into the Genie III LAMP machine, load a program for contest temperature of 42°C for 30 min.
- Samples mixed with pellet are transferred into the 8-well strips for the Genie III compatibility.
- Run the program
- Results were similar as seen in AmpliFire® on the Genie screen. Positive sample will produce “S” shaped amplification curve whereas for the negative result showed a flat line. Each sample has unique colour for differentiation on the screen. Genie machine also shows results for time to positive.

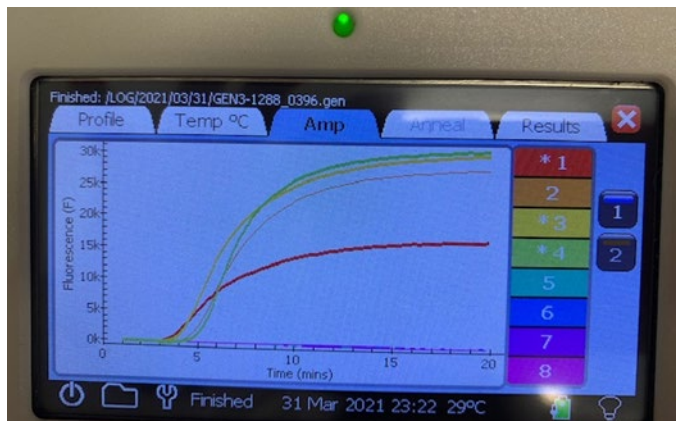


Figure 2. LAMP amplification for four positive (sigmoid) amplification curves (red, orange, yellow, green) and four negative amplifications (flat) (light blue, dark blue, purple and pink).

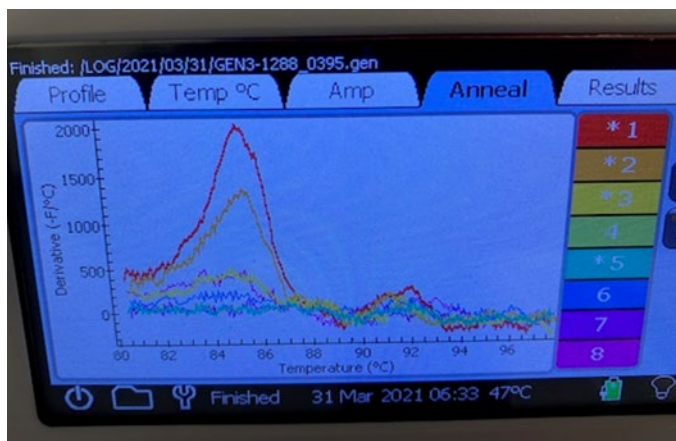


Figure 3. As a confirmation of the correct target, anneal derivates (AD) were generated by adding a melt stage to the program in Genie III machine. The AD is specific for a target based on GC content of the generated amplicon. Anneal derivates of the RPA reaction were observed for four positive samples but the first two sample, red and orange had higher peaks having higher concentration of the targets in a sample.

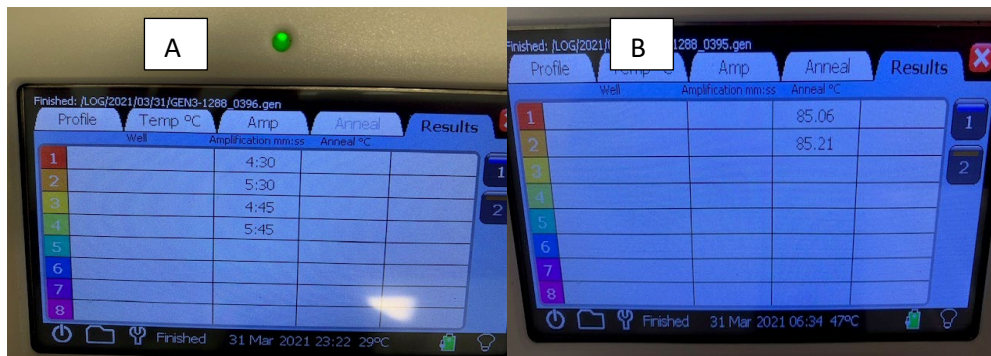
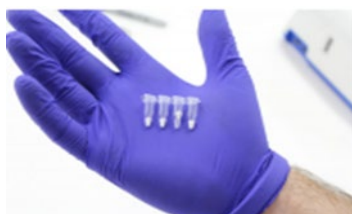


Figure 4. A - The time to positive can be seen in the Genie III machine similar to LAMP. The time to positive for samples 1 to 4 that produced an amplification curve were seen 4 min 30 sec (red), 5 min 30 sec (orange), 4 min 45 sec (yellow) and 5 min 45 sec (green). The earlier time to positive for sample 1 (well red) indicated there was a higher concentration of the target in that sample compared to other samples. B – Anneal derivative of the positive samples.

2. Endpoint detection using amplicon detection chambers.

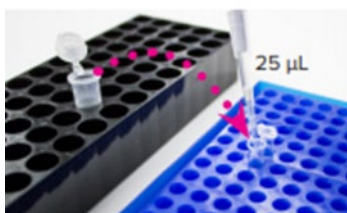
Amplification

1. Allow heat block to warm to 42 °C before preparing reactions. If using an Agdia-supplied heat block, allow 2 to 3 minutes for this step.
2. Remove the strip of reaction pellets from the desiccated container included in the kit. While securing the strip of pellets in a 200 µL PCR tube rack, cut the number of reaction pellets from the strip that are intended for use. Immediately place remaining reaction pellets back



into the desiccated tube for later use.

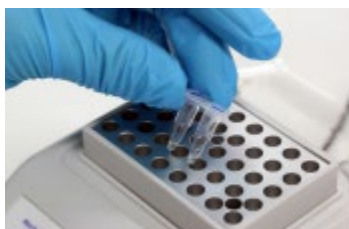
3. Transfer 25 µL from the microcentrifuge tube containing your sample extract into the reaction pellet (clear tube).



4. Tightly recap the reaction tube. Mix well and centrifuge. If you cannot vortex the reaction, mix by gently flicking the side of the tube. If you do not have a centrifuge available, you may manually shake the liquid to the bottom of the reaction tube.

Important: do not transfer more than the prescribed 25 µL during this step! immediately proceed to the next step once the reaction has been rehydrated.

5. Add reaction to the portable heat block for 4 minutes. After 4 minutes of incubation remove the reaction from the heat block. Quickly mix, spin, and reinsert the reaction into the heat block for an additional 16 minute



6. Immediately remove reaction from heat block and proceed to detection steps.

Detection

Note : In order to avoid possible contamination of future tests, do not open the reaction pellet.

Step 1. Open the foil pouch containing the Amplicon Detection Chamber (ADC). There are two pieces to the chamber as indicated in the figure 4 to the right.

Step 2. Add the unopened reaction tube to reaction apparatus as illustrated to the right (a). Once the tube has been added, snap the apparatus shut which will immobilize the reaction tube (b).

Step 3. Add the reaction apparatus to the detection chamber housing as indicated. IMPORTANT: The reaction tube should be facing toward the lateral flow strip, contained in the housing, during this step.

Step 4. Push down on the handle of the detection chamber housing until it snaps shut. Wait 20 minutes before interpreting results. Positive results may be visible in as little as 5 to 10 minutes. Samples that contain lower copy numbers may take up to 20 minutes to produce a positive test line.

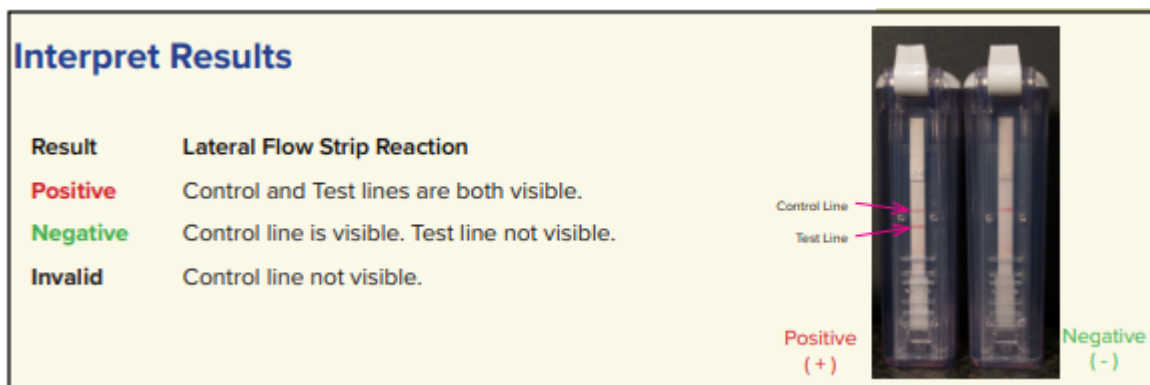
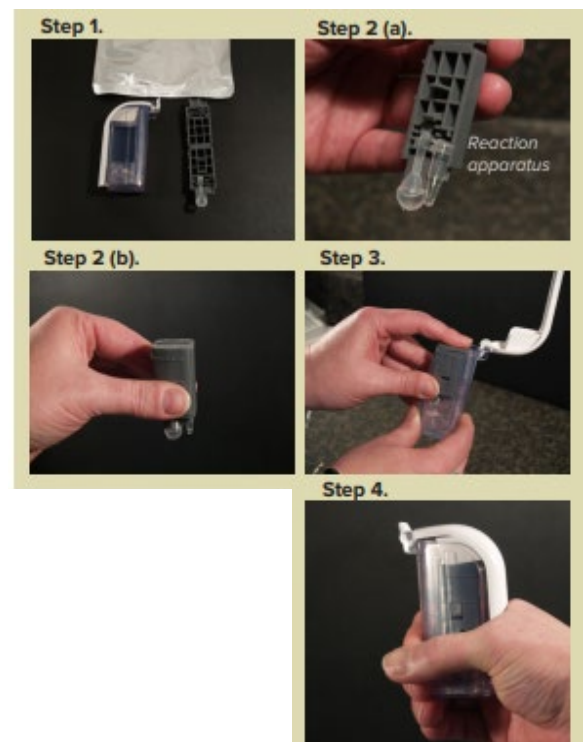


Figure 4. Step by step diagrams to insert the reaction tube into the lateral flow chambers.

Results

The chamber has lateral strips, the buffer starts to rise upwards as soon as chamber is shut closed. There was appearance to two lines for positive samples as shown in the interpret results box and in figure 5. The upperline is a control line where as lower line is the test line.

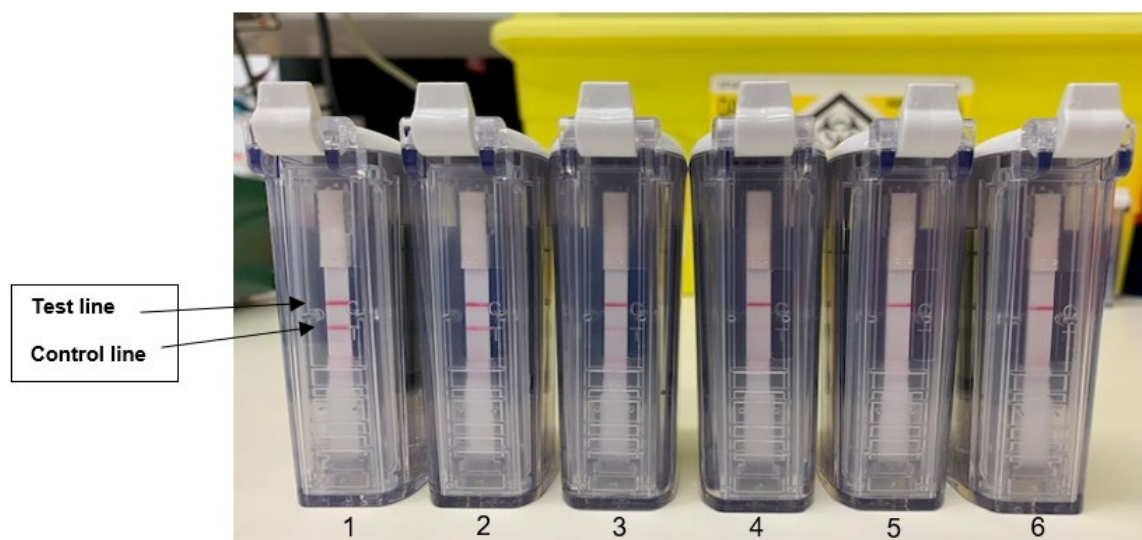


Figure 5. The lateral flow chambers 1-4, (4 with a faint test line) showing positive two lines whereas samples 5 and 6 were having a single control line as negative.

Precautions/ Limitations

Reaction Volume: Care should be taken to ensure the volume used to rehydrate the reaction is within $\pm 10\%$ of the prescribed $25\ \mu\text{L}$ mentioned in step 5 and 3 the Test Protocols. Deviating outside this tolerance may result in test failure.

Inhibition: Some plant species may cause inhibition with this assay if the sample extract is too concentrated. Please follow the extraction and sample dilution steps carefully. Addition of sample extract to reaction pellet: It is important to add only the prescribed amount of sample extract to the pellet diluent tubes. Adding too much extract may cause test failure.

Storage: Test results may be weak, or the test may fail if the storage instructions are not followed properly. The lyophilized test components must remain protected from light to prevent bleaching and sealed with desiccant when not in use to prevent moisture degradation, which may affect test results. Do not store pellets at temperatures greater than $42\ ^\circ\text{C}$, even for short periods of time, as this may cause test failure.

References

Hopkins, D. L. (1981). Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. *Phytopathology* 71, 415–418. doi: 10.1094/Phyto-71-415

Development of National Diagnostic Protocol and targeted sequencing approaches for *Xylella* spp identification in AUS and NZ

Dr Pragya Kant
Research Scientist



Hort
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Locations

Bundoora, Horsham, Hamilton
Tatura, Ellinbank

Livestock and animals

Crops and horticulture

Biosecurity

Climate and weather

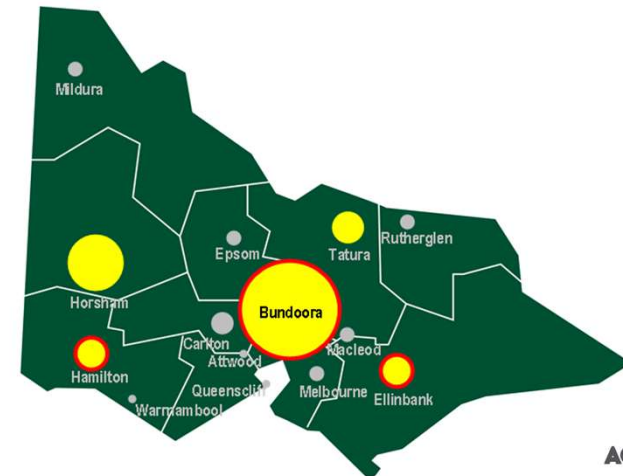
Farm management

Export

Support and resources

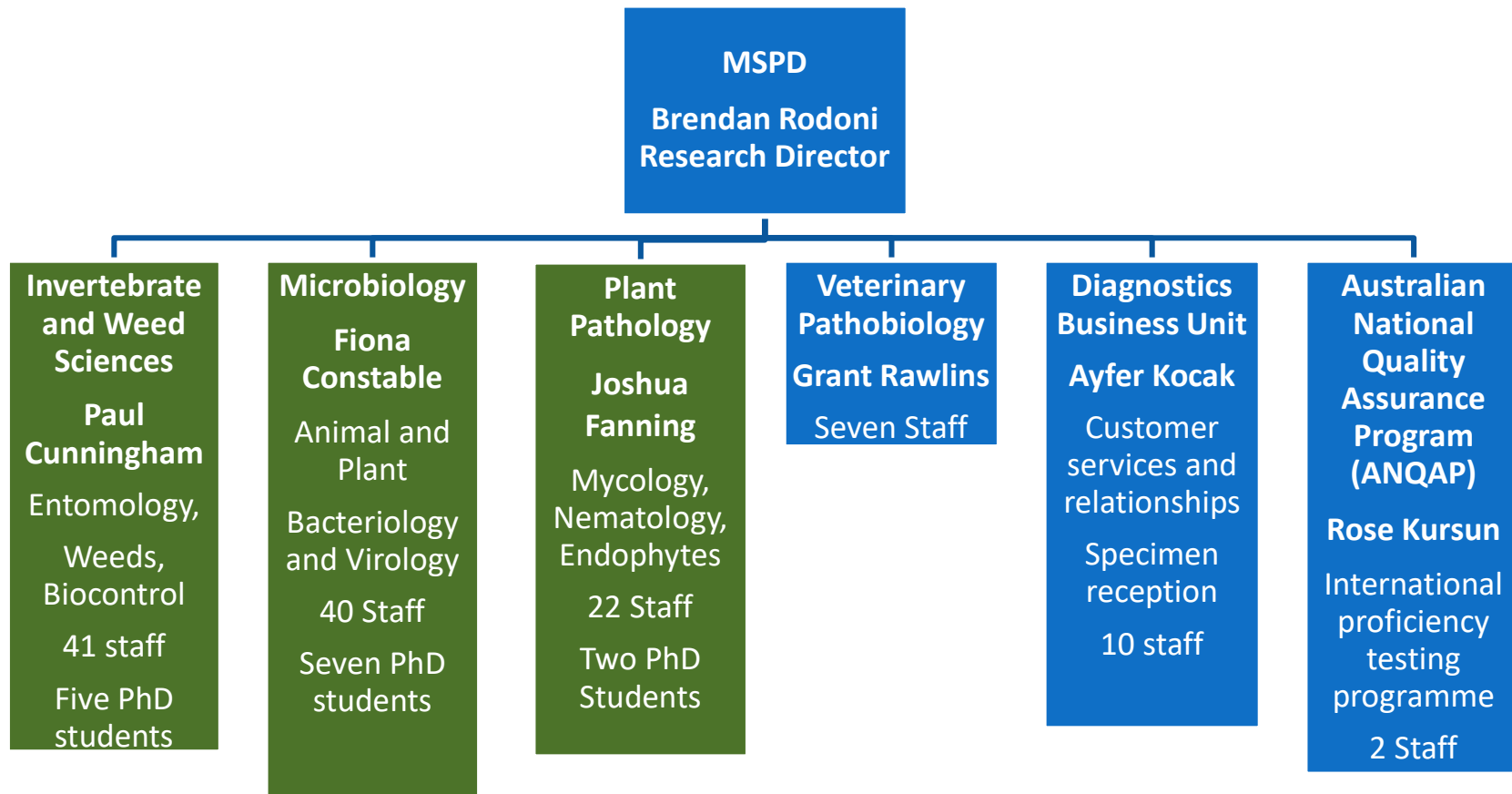


AgriBio Center of AgriBiosciences , Bundoora, Melbourne



AGRICULTURE VICTORIA

Microbial Sciences, Pests & Diseases



Fee-for-service diagnostic/biosecurity capacity in a NATA accredited laboratory
Enhancing response and management of plant and animal pest and disease outbreaks

- 125 staff

Why I came here?

- Australia and New Zealand doesn't have *Xylella*, no1 priority pest.
- We want to prepare ourselves if it gets detected
- Australia has economical crops such as Grapevines, Olives, Citrus, Almonds
- We do have spittlebugs native- one species is dominating
- We want our protocols for detection are efficient
- Learn from the countries who has *Xylella* and how they do diagnostics and surveillance.
- *Xylella* is hard to work with.

Current approach to *Xylella* diagnostics in Australia

- Annual surveillance in most states (includes vector monitoring around major ports, targeted surveillance (e.g. vineyards) and general/passive surveillance)
- Current Australian National Diagnostic Protocol (NDP) for *Xylella* is focused on **Pierce's disease.- OLD 2000 and 2016** (Harper et al 2010 qPCR and Minsavage et al 1994)
- New NDP for *Xylella* spp. was required.

Xylella Taxonomy

Two species: *Xylella fastidiosa* and *Xylella taiwanensis*

Subspecies: six different subspecies of *X. fastidiosa* have been proposed

- *X. fastidiosa* subsp. *fastidiosa*
- *X. fastidiosa* subsp. *multiplex*
- *X. fastidiosa* subsp. *pauca*
- *X. fastidiosa* subsp. *sandyi*
- *X. fastidiosa* subsp. *tashke*
- *X. fastidiosa* subsp. *morus*

sandyi & *morus* now considered part of *fastidiosa*, *tashke* is not isolated since identified

Further classified into Sequence types (ST). Sequence Typing is related to host specificity and behave like different diseases.

e.g. *Xf pauca*, infected citrus CVC and then infected Olives OQD

Sequence Types are important to know- a diagnostic gold !!

Collections/databases



Dr Luciano Rigano



Dr Robert Taylor



Dr Natasha



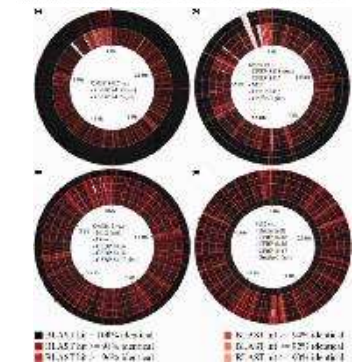
Dr Pragya

Establish a collection of *Xylella* isolates (**MPI NZ & AgVIC lead**)

- live cultures (currently only in NZ)
 - Hands on experience (media prep, culturing, identification)
 - Positive controls -22 live cultures Xf, 1 Xt,
 - 21 Xf infected plant samples, DNA extracts
- DNA
 - Positive DNA controls for Australian laboratories
 - Proficiency testing
- Genomic sequence database- illumina and Nanopore
 - Generated genomic sequence (>20 Xy isolates)
- Bioinformatics pipelines established – UBCG, mlst, stringMLST, NGspeciesID



Xylella fastidiosa ICMP8/15 on DCYL Dr Luciano Rigano MPI/NZ



Denancé, 2017 Plant pathology

High-throughput Sequencing for Improved and Rapid Diagnostics

- ✓ Cheaper to do HTS
- ✓ Time saving
- ✓ Applied directly on host samples
- ✓ More data means more information
- ✓ Handle large number of samples

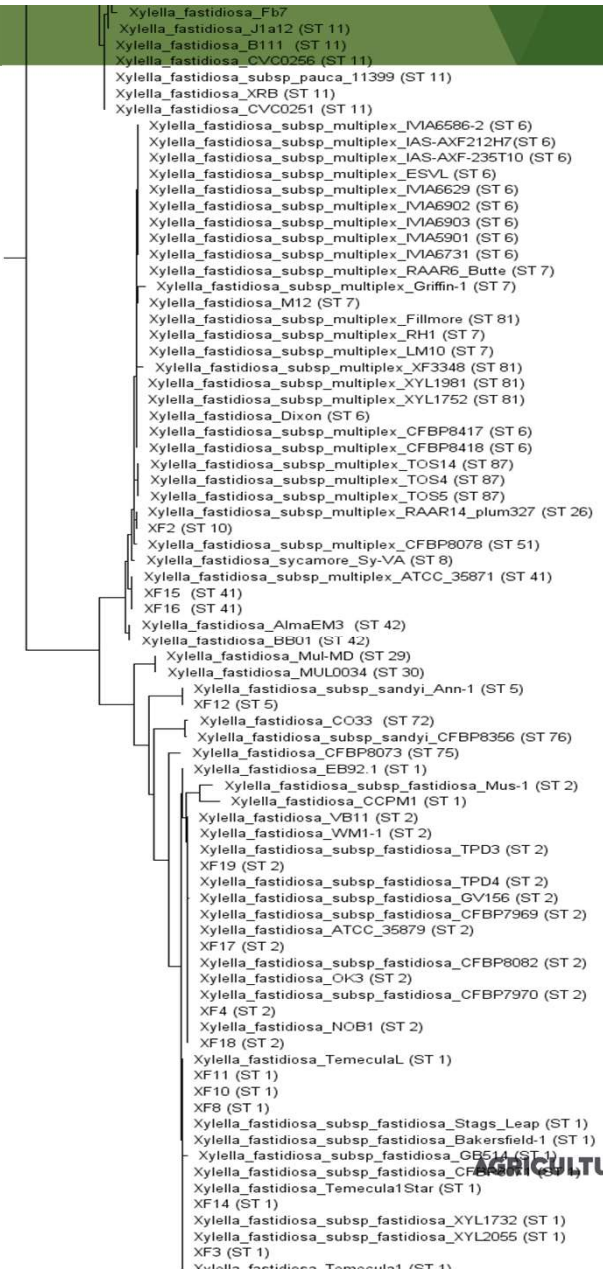
ST assignments using third-generation sequencing platforms:

- Illumina WGS, core genome to get ST (UBCG)
- Oxford Nanopore Technologies- amplicon MLST sequencing directly from plants
- Assign ST from metagenomic data (StringMLST, MetaMLST, MLST, PubMLST)



Up-to-date-Bacterial Core Genome

- Publicly available pipeline: UBCG- (up-to-date bacterial core gene set)- Na et al. 2018
- Uses 92 core genes- bacteria and archaea
- Implement as part of the standard HTS pipeline for bacterial ID in our lab
 - No need for individual genus-based databases e.g. MSLT/MLSA
 - No PCR bias
- 115 *Xylella* genomes- sequence and NCBI
 - Accurate subspecies differentiation
 - ST clustering



Lab-based methods approved for NDP

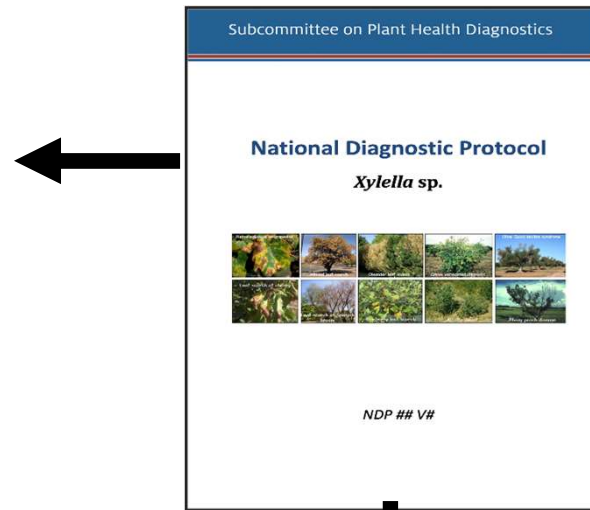
- qPCR for Xf by Harper et al 2010
- qPCR for Xf by Dupas et al 2019
- Generic qPCR *Xylella* spp. and Xt (Newly developed)
- Duplex qPCR with Harper assay and XT specific probe (Newly developed)
- Endpoint PCR generic *Xylella* spp. assay coupled with sequencing (Newly developed)
- 5 laboratories- NZ, VIC, WA, NSW, QLD
- Specificity DNA panel, sensitivity panel of Xt and Xf subspecies Xff, Xfm, Xfp
- Proficiency testing on blind samples for both wet and dry lab methods across labs



Dr Toni Chapman and
Dr Johanna Wong
NSW DPI

Updated NDP

Processing of samples & isolation of bacterial cultures



Section 9 – surveillance samples and in-field assays

Generic *Xylella* Endpoint PCR assay, with sequence confirmation

Xylella qPCR assay

Differentiation between *X. fastidiosa* and *X. taiwanensis*

Molecular assays

Subspecies identification multiplex qPCR

Genome sequencing

Multi-locus Sequence Typing (MLST)

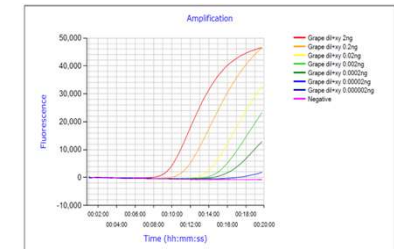
In-field detection of *Xylella*

LAMP by Harper et al 2010 and RPA- Agdia kit were recommended that can

- Detects both *Xf* and *X. taiwanensis*
- Detect all subspecies of *Xf*
- Is easy to use and interpret
- Specificity
- Sensitivity

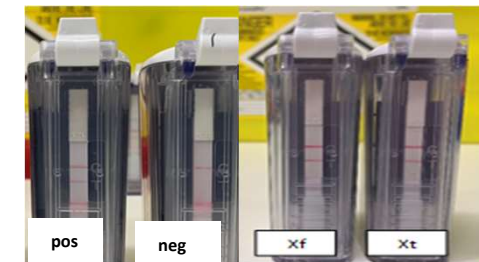


Dr Rachel Mann and Dr Pragya Kant



Sample Name	Xylella Conc. (ng/μL)	Harper qPCR CT Mean	Harper LAMP Tp	Agdia RT-RPA (Tp)	Agdia LFD - RPA
O+Xf1	0.2375	21.1	9.9	4:30	+ve
O+Xf2	0.02375	24.5	11.2	4:45	+ve
O+Xf3	2.375x10 ⁻³	27.9	12.9	5:30	+ve
O+Xf4	2.375x10 ⁻⁴	31.2	14.7	5:45	positive
O+Xf5	2.375x10 ⁻⁵	34.2	20.4	UD	
O+Xf6	2.375x10 ⁻⁶	37.5	UD	UD	
O+Xf7	2.375x10 ⁻⁷	UD	UD	UD	
O+Xf8	2.375x10 ⁻⁸	UD	UD	UD	

UD- Undetermined



Specificity Panel

- 31 *Xylella*
- 16 *Xanthomonas*
- 9 *Stenotrophomonas*
- 18 *Xylella* positive plant samples
- 1 healthy plant sample
- 6 *Xylella* positive insect samples

Sensitivity Panel

- *Xylella fastidiosa* subsp *fastidiosa*
- *Xylella fastidiosa* subsp *multiplex*
- *Xylella fastidiosa* subsp *pauca*
- *Xylella taiwanensis*

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Name	ID Code
<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 8731
<i>Xylella fastidiosa</i> subsp <i>multiplex</i>	ICMP 8739
<i>Xylella fastidiosa</i> subsp <i>multiplex</i>	ICMP 8740
<i>Xylella fastidiosa</i>	ICMP 8742
<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 8745
<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 15197
<i>Xylella fastidiosa</i> subsp. <i>piercei</i>	NCPB4432 / ICMP 15197
<i>Xylella fastidiosa</i> subsp. <i>multiplex</i>	NCPB4604 / BB164
<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8495 / PD7202
<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	CFBP8071
<i>Xylella fastidiosa</i> subsp. <i>sandyi</i>	CFBP8524 / C033
<i>Xylella fastidiosa</i>	CFBP8173
<i>Xylella fastidiosa</i>	CFBP7969
<i>Xylella fastidiosa</i>	CFBP7970 / ICMP 15197
<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8477 / Salento-1
<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8072
<i>Xylella fastidiosa</i>	CFBP8073
<i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i>	CFBP8082 / ICMP 8745
<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8498 / PD7211
<i>Xylella fastidiosa</i>	NCPB4605 / Temecula1
<i>Xylella fastidiosa</i> subsp. <i>sandyi</i>	CFBP8077 / Ann-1
<i>Xylella taiwanensis</i>	NCPB4612
<i>Stenotrophomonas</i> sp.	DAR 65801
<i>Stenotrophomonas</i> sp.	DAR 72045
<i>Stenotrophomonas</i> sp.	DAR 75512
<i>Stenotrophomonas</i> sp.	DAR 76132
<i>Stenotrophomonas</i> sp.	DAR 77232
<i>Stenotrophomonas</i> sp.	DAR 77233
<i>Stenotrophomonas</i> sp.	DAR 77234
<i>Stenotrophomonas</i> sp.	DAR 77236
<i>Stenotrophomonas</i> sp.	DAR 77237
<i>X. fuscans</i> subsp <i>aurantifolii</i>	1622 B Strain
<i>X. alfalfae</i> subsp <i>citrumelo</i>	P03- 83
<i>X. vasicola</i>	DAR41379
<i>X. vesicatoria</i>	DAR73877
<i>X. translucens</i> pv <i>translucens</i>	DAR35705
<i>X. hortorum</i>	DAR49849
<i>X. arboricola</i>	DAR33337
<i>X. campestris</i> pv <i>phaseoli</i>	DAR82645
<i>Xanthomonas</i> sp.	DAR82580
<i>X. oryzae</i>	DAR82611
<i>X. campestris</i> pv <i>vitians</i>	DAR30526
<i>X. campestris</i>	VPRI41552
<i>X. campestris</i> pv <i>pruni</i>	DAR82627
<i>Xanthomonas</i> sp.	USA 3- 8A.1
<i>X. caroliniana</i>	DAR82711
<i>Xanthomonas</i> sp.	DAR72015

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New Zealand	Victoria	New South Wales	Queensland	Western Australia	Northern Territory	South Australia	Norfolk Island
<i>Rosmarinus sp.</i> (Rosemary)	<i>Alnus sp.</i>	<i>Citrus sinensis</i> (Orange)	<i>Citrus aurantifolia</i> (Tahitian lime)	<i>Citrus limon</i> (Lemon)	<i>Citrus limon</i> (Lemon)	<i>Citrus reticulata</i> (Mandarin)	<i>Prunus</i> (Peach)
<i>Prunus armeniaca</i> (Apricot)	<i>Canna sp.</i>	<i>Citrus limon</i> (Lemon)	<i>Rubus idaeus</i> (Raspberry)	<i>Citrus spp.</i> (Limes)	<i>Citrus aurantifolia</i> (Tahiti lime)	<i>Citrus sinensis</i> (Orange)	<i>Vitis sp</i> (Grape)
<i>Prunus domestica</i> (Plum)	<i>Euphorbia sp.</i>	<i>Citrus reticulata</i> (Mandarin)	<i>Citrus australasica</i> (Finger Lime)	<i>Citrus sinensis</i> (Orange)	<i>Fortunella sp</i> (Kumquat)	<i>Citrus reticulata</i> 'Shiranui' (Dekapon)	<i>Prunus persica</i> (Peach)
<i>Prunus persica</i> (Plum)	<i>Ginkgo sp.</i>		<i>Citrus hystrix</i> (Kaffir lime)	<i>Citrus aurantifolia</i> (Tahiti lime)			<i>Citrus limon</i> (Lemon)
<i>Vitis sp.</i> (Grapevine)	<i>Nandina sp.</i>		<i>Citrus reticulata</i> (Mandarin)	<i>Olea europaea</i> (Olive)			<i>Persea americana</i> (Avocado, Hass)
<i>Quercus sp.</i> (Oak)	<i>Quercus sp.</i>		Tahiti lime x mandarin	<i>Vaccinium sect.</i>			<i>Vitis sp</i> (Grape grown from seed)
<i>Acer pseudoplatanus</i> (Sycamore maple)							<i>Macadamia integrifolia</i> (Macadamia)
<i>Liquidambar sp.</i> (Sweetgum)							<i>Prunus domestica</i> (Plum)
<i>Polygala myrtifolia</i> (Milkwort)							<i>Pyrus pyrifolia</i> (Pear)
<i>Olea europaea</i> (Olive)	<i>Portulaca sp.</i> (Portulaca)		<i>Macadamia integrifolia</i> (Macadamia)	<i>Fragaria x ananassa</i> (Strawberry)			<i>Vitis sp</i> (Grape)
<i>Platanus acerifolia</i> (London Plane)	<i>Pelargonium sp.</i> (Pelargonium)		<i>Citrus limon</i> (Lemon)	<i>Citrus x aurantiifolia</i> (Lime)			<i>Citrus reticulata</i> (Mandarin)
<i>Acer sp.</i> (Maple)	<i>Salvia Rosmarinus</i> (Rosemary)		<i>Citrus x aurantiifolia</i> (Lime)	<i>Prunus dulcis</i> (Almond)			<i>Prunus</i> (Stone fruit)
	<i>Lavandula sp</i> (Lavender)		<i>Mangifera indica</i> (Mango)	<i>Nerium oleander</i> (Oleander)			<i>Citrus limon</i> (Bitter lemon)
	<i>Vitis sp</i> Grapevine		<i>Litchi chinensis</i> (Lychee)	<i>Citrus reticulata</i> (Mandarin)			<i>Moraceae</i> (Fig)
	Citrus spp.		<i>Olea europaea</i> (Olive)	<i>Macadamia integrifolia</i> (Macadamia)			<i>Citrus x sinensis</i> (orange)
			<i>Citrus maxima</i> (Pomelo)	<i>Quercus</i> (Oak)			<i>Nicotiana glauca</i> (Tobacco)
			<i>Murraya koenigii</i> (Curry leaves)	<i>Acer</i> (Maple)			<i>Malus domestica</i> (Apple)
			<i>Eugenia uniflora</i> (Brazilian cherry)	<i>Citrus x paradisi</i>			<i>Prunus salicina</i>

Total of 403 plant samples were tested that covered 49 genera from AUS and NZ

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AGRICULTURE VICTORIA



Nanopore in diagnostics

Pragya Kant

Research scientist

AGRICULTURE  VICTORIA

High Throughput Sequencing (HTS) facility at AVR

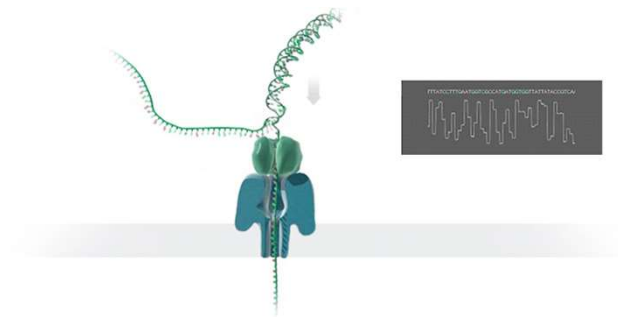
- Next-Generation Sequencing (NGS) platforms: Illumina, Nanopore, and PacBio.
Fast, sensitive, accurate, cheaper
- BASC- Biosciences Advanced Scientific Computing
- Conducting big sequencing based projects such as DairyBio, Pulse breeding programs, Microbiome
- Plant pathogens bacteria, fungus, viruses and viroids, phytoplasma as well as metabarcoding of insects pest, iMapPEST



Advantages of Nanopore sequencing

- High-throughput third generation sequencing platform.
- Fast and quick access to data, real-time data output
- Smallest, portable instruments which has lowest instrument and consumables costs.

long DNA molecules → long read length



Electric signals are recorded and later converted into bases

Longest confirm reads >100kb

Many instruments to choose from

<https://nanoporetech.com/>

Sequencing instruments



Cheap \$90.
Great for
fungal and
bacterial work.
Data-500Mb
Single use
Run time 16hr



Good option.
Great for
fungal and
bacterial work.
Data-20Gb
Multiple use
Multiplexing
Run time 72hr



Everything
you need in
one device
for MinION



5 minion,
100 Gb data
real-time
Run time
72hr



24 to 48
flowcells.
Lowest price
per Gb of data
150Gb data
per flowcell
Run time 64hr



On horizon-
smallest seq
device to use
with smart
phone in any
location

MinkNOW program is required to run all these devices

Computational and bioinformatics requirements

GPU works better for the base calling than CPU

A fast processor such as intel i9 with multiple nodes

Hard drive 1TB

RAM 64 to 128 GB

- Bioinformatics tools specific for long reads analysis
 - Guppy
 - Nanoplot
 - Unicycler
 - filtlong
 - Flye
 - Racon
 - Medaka
 - Kaken2



<https://usegalaxy.org.au/>

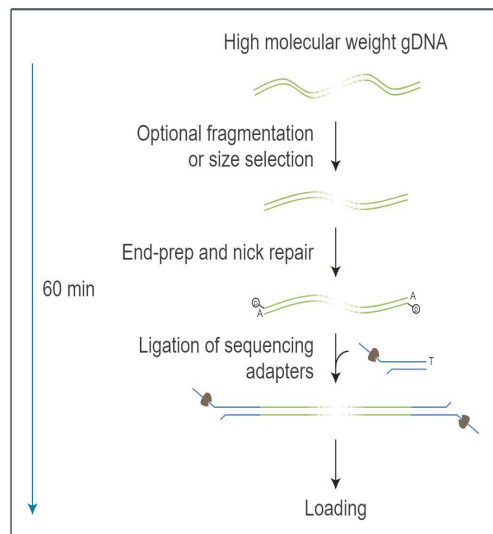
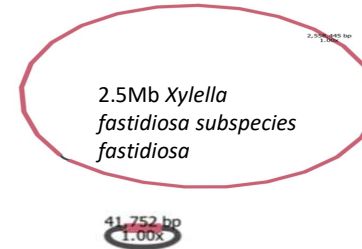
**1. Whole genome sequencing –
pure bacterium culture**

2. Amplicon sequencing

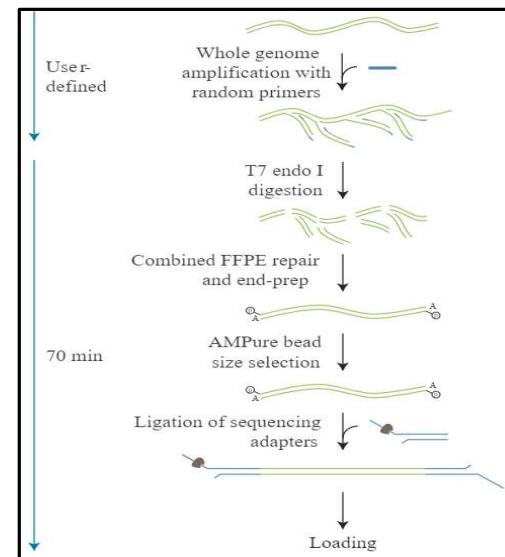
Whole genome sequencing

Input DNA

Extraction methods



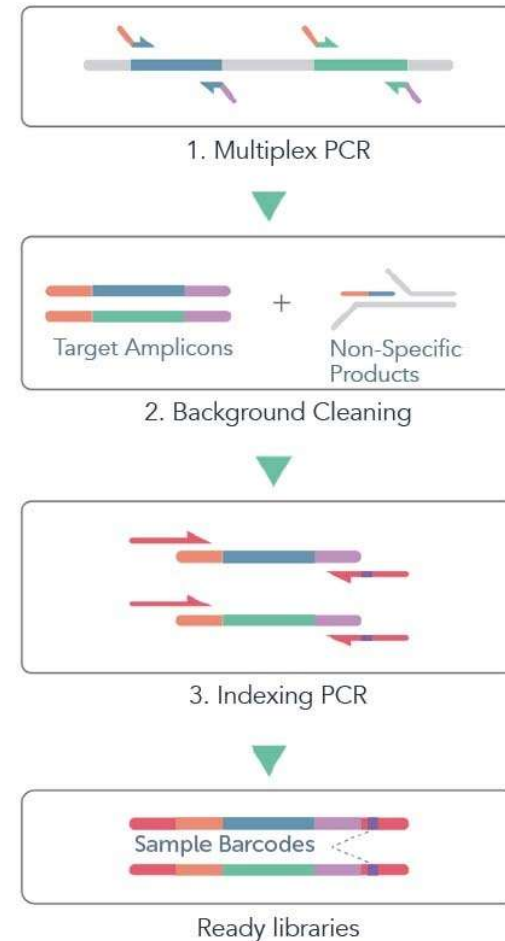
Genomic DNA sequencing



Whole genome amplification sequencing

Amplicon sequencing

- Offers a fast detection tool
- Amplicons are targeted and highly specific regions, accurate detection
- Directly applicable on metagenomic samples and samples with low titre pathogens
- Approach is much sensitive than qPCR
- Amplicon sequencing doesn't need much data 1-2 hrs of run is sufficient
- Easily applied on MLST/serotypes/ barcoding

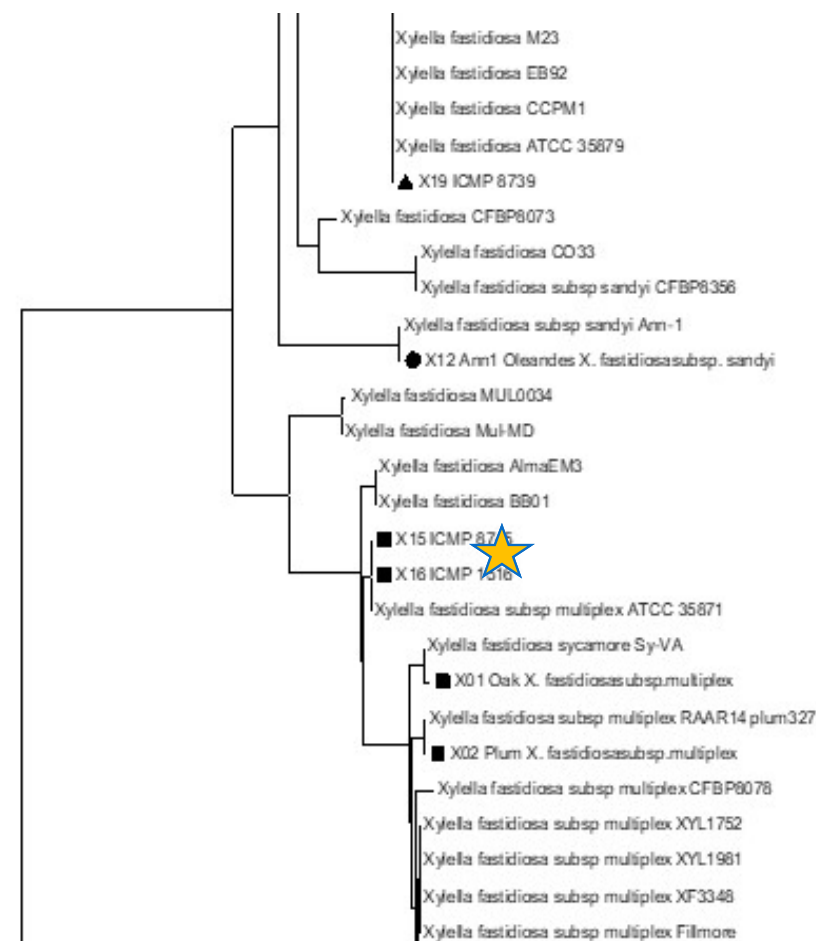


Whole genome sequencing-

1. Polishing using Illumina reads

MinION only				
Sample	Total length	Assembly	plasmid	ST
ICMP 8739	2,568,573	incomplete	no	0
ICMP 8740	2,547,368	complete	no	1
ICMP 8731	2,569,483	complete	yes	1
ICMP 15197	2,636,217	complete	yes	1
ICMP 8742	2,597,866	complete	yes	2
ICMP8745	2,729,604	incomplete	no	1

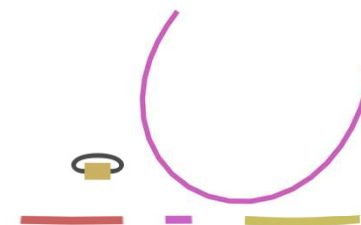
MinIon and Illumina				
Sample	Total length	Assembly	plasmid	ST
ICMP 8739	2,524,685	complete	no	41
ICMP 8740	2,548,838	complete	no	41
ICMP 8731	2,561,742	complete	yes	1
ICMP 15197	2,600,197	complete	yes	2
ICMP 8742	2,598,507	complete	yes	2
ICMP8745	2,645,299	complete	yes	2



Dealing with errors-

2. Extracting high quality reads

	Before subset	After sub-set
Number of reads	332,224.00	47,999
Total base	1,059,284,840.00	500,000,739
Median read length	1,844.00	9,005
N50	6,113.00	10,853
>Q7:	331291 (99.7%)	47999 (100.0%)
>Q10:	295655 (89.0%)	46573 (97.0%)
>Q12:	235588 (70.9%)	40447 (84.3%)
>Q15:	88804 (26.7%)	14765 (30.8%)



Whole genome assembly- Multiple contigs

Depth of chromosome coverage	Taxonomy					MLST														
78X	X. fastidiosa subspecies fastidiosa					ST2														
																				
<table><tr><th>cycG</th><th>gltT</th><th>holC</th><th>leuA</th><th>petC</th><th>malF</th><th>nouL</th></tr><tr><td>1</td><td>1</td><td>1</td><td>1</td><td>1</td><td>4</td><td>1</td></tr></table>							cycG	gltT	holC	leuA	petC	malF	nouL	1	1	1	1	1	4	1
cycG	gltT	holC	leuA	petC	malF	nouL														
1	1	1	1	1	4	1														

High Throughput Sequencing (HTS) for pathogen detection (metagenomic)

DNA of *Xylella fastidiosa* subsp. *fastidiosa* (Xff) was serially diluted into olive DNA.

LAMP, qPCR and metagenomic sequencing was run on Xff standards.

- Results showed qPCR was 1000-10,000 times more sensitive than metagenomic studies (<10GB)
- Identification of Xff in GB

Limitation – Host DNA is predominately sequenced- waste of resources, specifically for low-titre, unculturable and fastidious bacteria

Way-out is Enrichment- Probes and Amplicon seq

Concentration of <i>Xylella</i> DNA (ng)	24.5	11.2	XF	XF	ST2	2089	4686	9542	133	488	1233	690	1570	3106	1.84	7.23	20.59
2.4 x 10 ⁻⁰²	24.5	11.2	XF	XF	ST2	2089	4686	9542	133	488	1233	690	1570	3106	1.84	7.23	20.59
2.4 x 10 ⁻⁰³	27.9	12.9	UD	UD	XF	190	501	1155	3	11	45	523	439	575	0.05	0.142	0.619
2.4 x 10 ⁻⁰⁴	31.2	14.7	UD	UD	UD	13	57	96	1	1	1	317	281	333	0.012	0.011	0.013
2.4 x 10 ⁻⁰⁵	34.2	20.4	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁶	37.5	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁷	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁸	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

AVR has developed two targeted strategies using HTS

- Hybridization Probe – applicable at the PEQ to target National Plant Priority Pest list

Xylella spp

Erwinia amylovora

Phytoplasmas

Xanthomonas citri subsp. *citri*

Liberibacter

- Amplicon HTS – applicable at the time of surveillance and an outbreak of *Xylella fastidiosa* detection and sequence typing

Both methods have been applied for *Xylella* spp. detection with increased or equivalent sensitivity than a qPCR

High Throughput Sequencing (HTS) for bacterial pathogen detection

- Aim was to use HTS to detect all phytopathogenic bacteria on the National Priority Plant Pests (NPPP) list in one test (consisting of 12 species/subspecies across 5 genera)
- Many of these are low titre, fastidious, non-culturable
- Multiple PCRs/Sanger sequencing for detection and identification
- What do we want from using HTS:-
 - prof of concept
 - Detection of all pathogens in a sample
 - Retain or improve sensitivity of current tests
 - Make cost effective



Erwinia amylovora



Xanthomonas citri
subsp. *citri*



Xylella
fastidiosa



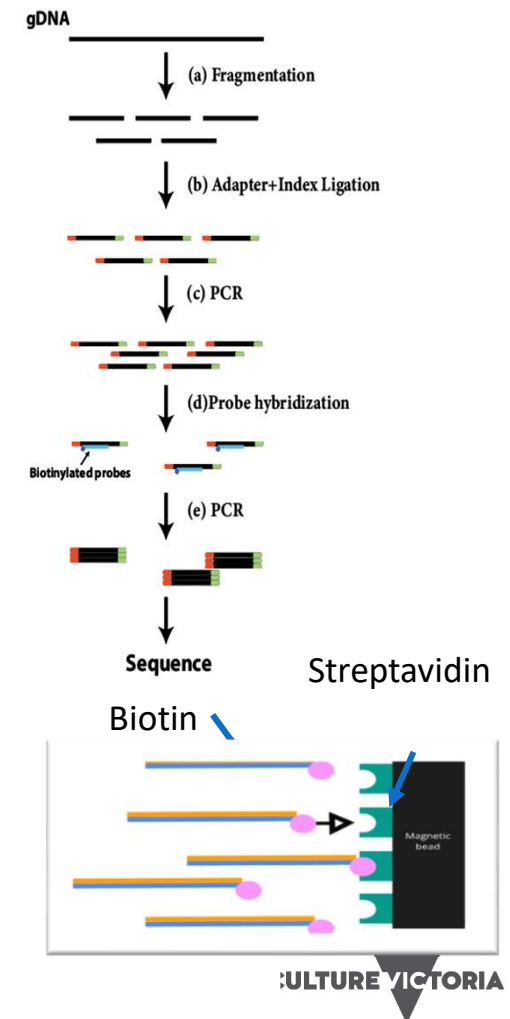
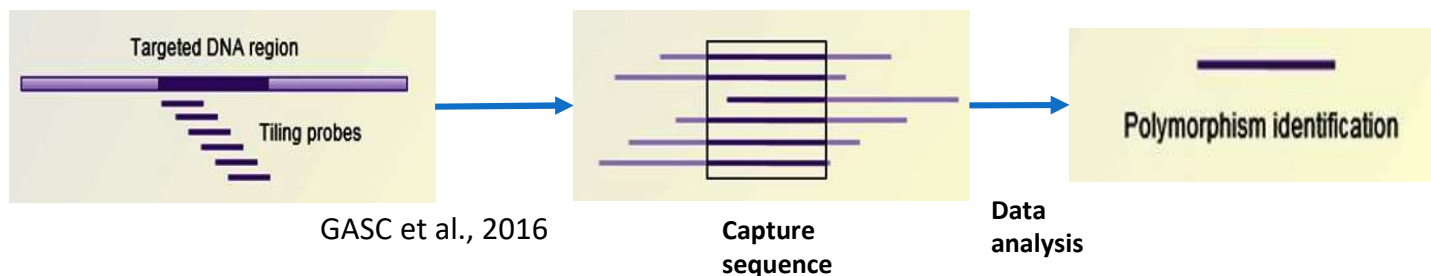
Candidatus
phytoplasma
16SrI



Ca. *Liberibacter*
asiaticus

High Throughput Sequencing (HTS) for pathogen detection

- Literature and databases were searched and compared for putative target regions
 - MLSA/MLST genes
 - Diagnostic target regions
 - Genes associated with biosynthetic pathways
 - 3071 probes specifically for *Xylella*
 - 15000 probes for 5 genera



Increased sensitivity to the target regions in metagenomic samples

Sample Name	qPCR	<i>Xylella fastidiosa</i> subsp.	Probed	On-target reads	On-target percentage (%)
Vitis B1-2	23	<i>fastidiosa</i>	Yes	1292502	47.31
			No	215	0.0041
Vitis B2-3	24	<i>fastidiosa</i>	Yes	5767959	44.72
			No	211	0.0023
Vitis B2-7	30	<i>fastidiosa</i>	Yes	436552	36.05
			No	124	0.0013
Olea B3-1	23.7	<i>sandyi</i>	Yes	19887876	64.44
			No	65	0.0013
Olea B4-2	25.3	<i>sandyi</i>	Yes	12970131	60.93
			No	29	0.0008
NGS XF Vitis-1	24.6	<i>fastidiosa</i>	Yes	17043697	59.35
			No	76	0.0020
NGS XF Vitis-2	26.7	<i>fastidiosa</i>	Yes	22215079	59.73
			No	73	0.0014
Spiked ICMP 8739	27.04	<i>multiplex</i>	Yes	2273256	57.74
			No	129	0.0016
Spiked CFBP 8072	30.57	<i>pauca</i>	Yes	248600	26.11
			No	18	0.0002
Spiked NCPPB 4612	-	<i>Xylella taiwanensis</i>	Yes	263290	41.30
			No	15	0.0002

Sensitivity of hybridization probes - XFF

Concentration of Xff DNA (ng/ μ L)	LAMP (amp time)	Non-probed		Probed		Fold enrichment	qPCR (Ct)
		Detected	MLST	Detected	MLST		
0.24	9.9	D	ST	D	ST	5,608	21.09
2.4×10^{-02}	11.2	D	XF ¹	D	ST	84,370.4	24.52
2.4×10^{-03}	12.9	-	-	D	ST	122,206.3	27.87
2.4×10^{-04}	14.7	-	-	D	ST	23,597.2	31.17
2.4×10^{-05}	20.4	-	-	D	ST	45,796.9	34.21
2.4×10^{-06}	UD	-	-	D	XF ¹	1,963.5	37.45
2.4×10^{-07}	UD	-	-	-	-	*	UD
2.4×10^{-08}	UD	-	-	-	-	0.6	UD

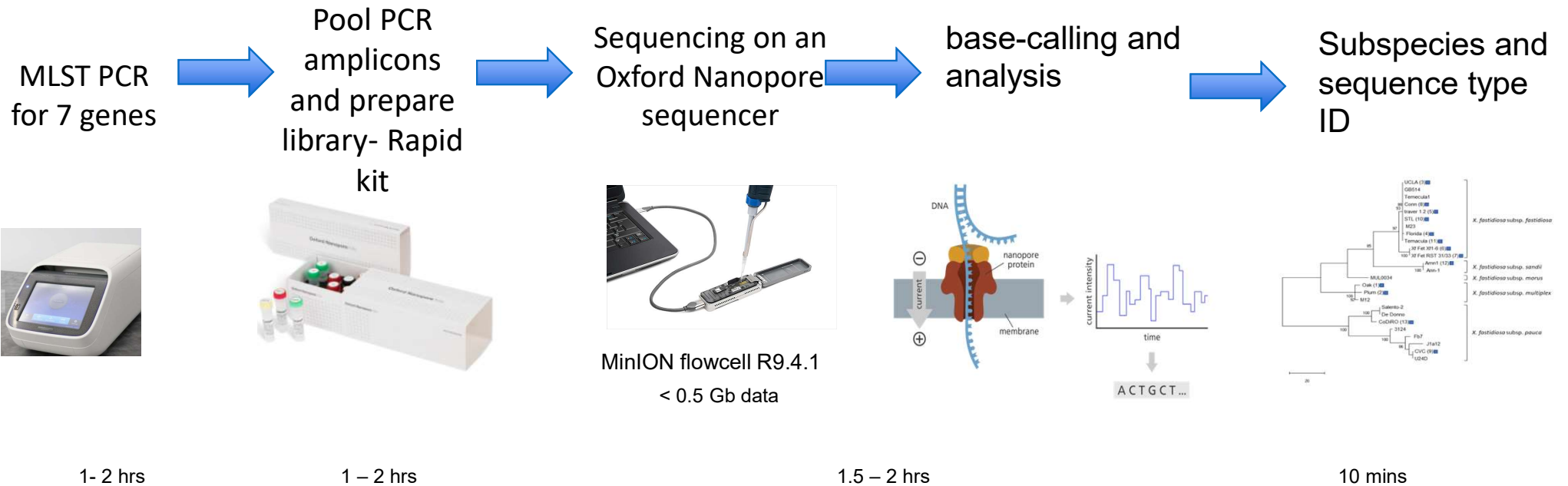
- Same level as qPCR
- Identification of ST at Ct 34.2

Multi-locus sequence typing- To characterise *Xylella fastidiosa*

- 7 genes for *X. fastidiosa* *cys*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC* (Sclly et al 2005)
- Different alleles for each gene- 156 alleles 90 known STs (www.niuhmlet.org)
- Exam **In case of an incursion- it is valuable to** trees
in So **know Sequence Type ASAP**
- *Xf pauca* (main hosts: Citrus and Citrus spp.)
- STs are essential as they are associated with host specificity and introduction

Detection of *Xylella* – MLST amplicon sequencing

Similar work flow to Fiano et al. 2019



Completed in a day

Increased sensitivity of detection with enrichment

Spiked concentration of Xf DNA in Olive		qPCR	LAMP	Metagenomic seq			Enrichment	
		Haper et al 2010	Harper et al 2010 (Tp)	2 GB	5 GB	10 GB	MLST-amplicon seq	Hyb. Probes
No dilution	240 pg/ μ l	21.90	9.9	ST2	ST2	ST2	ST	ST
10 ⁻¹	24 pg/ μ l	24.52	11.2	XF	XF	ST2	ST	ST
10 ⁻²	2.4 pg/ μ l	27.87	12.9	UD	UD	XF	ST	ST
10 ⁻³	240 fg/ μ l	31.17	14.7	UD	UD	UD	ST	ST
10 ⁻⁴	24 fg/ μ l	34.21	20.4	UD	UD	UD	ST	ST
10 ⁻⁵	2.4 fg/ μ l	37.45	UD	UD	UD	UD	ST	XF
10 ⁻⁶	240 ag/ μ l	UD	UD	UD	UD	UD	XF	UD
10 ⁻⁷	24 ag/ μ l	UD	UD	UD	UD	UD	UD	UD
10 ⁻⁸	2.4 ag/ μ l	UD	UD				UD	UD

HTS combined with enrichment is a powerful tool for rapid and robust detection of low-titre and fastidious pathogens

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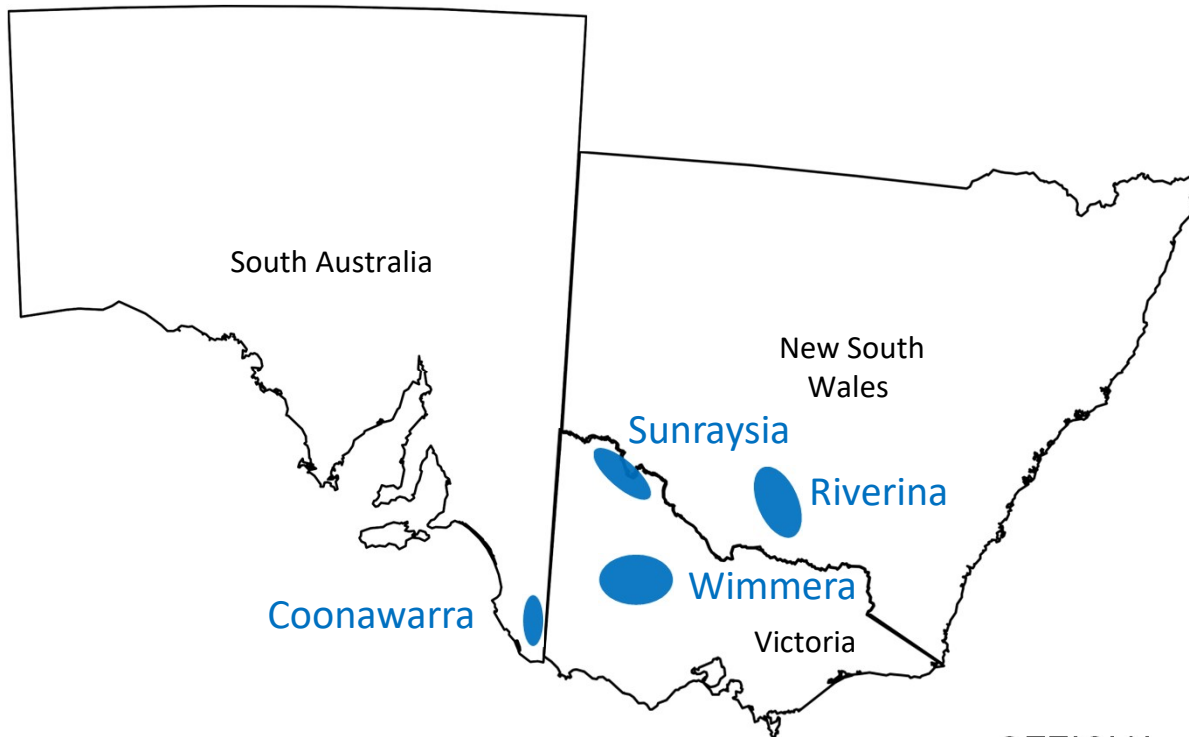
***Xylella* vectors project will support the development of effective vector monitoring, surveillance and control strategies for *X. fastidiosa* by:**

- Identifying potential vectors (obligate xylem feeders) of *Xylella* in Australian plant industries at risk
- Determining the critical times of the year when potential vector populations peak and the spread of *Xylella* can be most rapid
- Understanding of the biology and population dynamics of potential vectors
- Understanding the feeding behavior and plant host range of potential vector species
- Update the revised National Diagnostic Protocol for *Xylella* to include a vector component for isolation of pathogens and molecular analysis of samples.

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Xylella vectors project aimed to

Provide the tools and knowledge to mitigate the potential spread of *Xf* in Australia by understanding biology and population dynamics of potential vectors of *Xf* in grapevines, olives and citrus plantations.

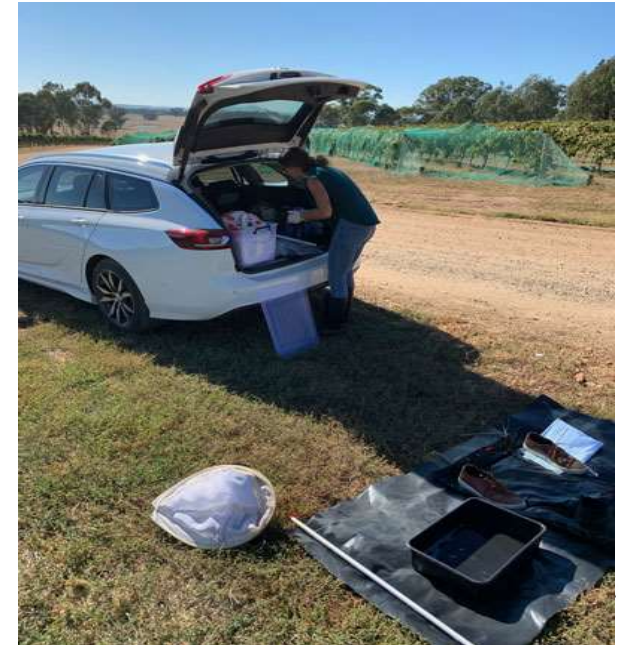


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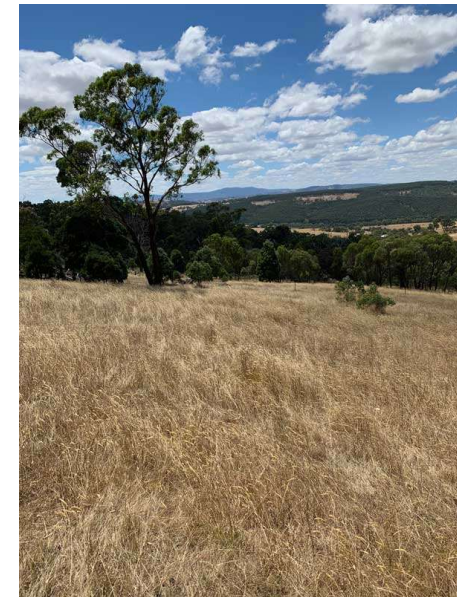
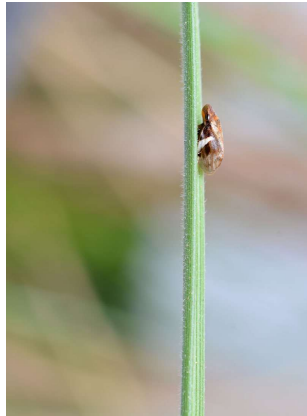
Surveillance methods



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Bathyllus albicinctus native spittlebug



Natasha Brohier
Rachel Mann
Brendan Rodoni
Fiona Constable
Elisse Nogarotto
Bianca Rodrigues Jardim
Tongda Li

Mark Blacket, Cait J Selleck,
Jackson Batchelor

Rebecca Roach
Monika Kehoe
Toni Chapman
Johanna Wong

Luciano Rigano
Robert Taylor



Ministry for Primary Industries
Manatū Ahu Matua



**Hort
Innovation**



**Wine
Australia**



Department of
Primary Industries and
Regional Development



Australian Government
Department of Agriculture,
Water and the Environment



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Thank you
Dr Burbank and USDA
Xylella team!!!!

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Field activity preparation

Xylella NDP:

LAMP and Agdia assays

Lucy Tran-Nguyen and Pragya Kant

ADSW 2023
9-11 MAY | CANBERRA

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Xylella

- *Xylella* spp are not present in Australia or New Zealand
- *Xylella* is Australia's number one on National Priority Plant Pest list:
 - Two *Xylella* species:
 - *Xylella fastidiosa* (>600 host species)
 - *Xylella taiwanensis* (only reported on *Pyrus pyrifolia*)
- In-field diagnostics:
 - Easily deployable assays that can be used in-field for rapid results
 - Can be used as a triage tool on symptomatic plants as part of surveillance



Photo: D. Boscia PLOS Biology 2018



Photo: Fiona Constable - Almond leaf scorch

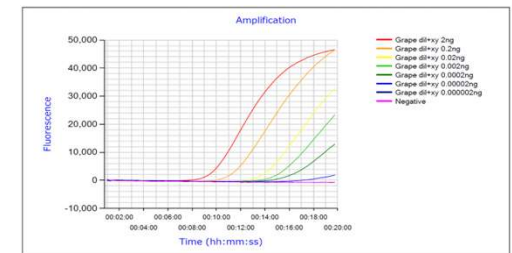


Temecula vineyard (2000) destroyed by Pierce's disease.

In-field detection of *Xylella* - Isothermal

Goal: In-field detection capability ready for application in the field by field officers

- Isothermal in-field assays (LAMP and RPA) identified as the most field ready technology and transferable to field officers
- Six different isothermal assays were assessed in an Australian setting
 - Specificity
 - Sensitivity
 - Ease of use by operator
- Recommendation for inclusion in the NDP:
 - LAMP of Harper et al (2010)
 - Agdia Amplify RP® XRT kit
 - Both assays were extensively validated by AgVic and can detect both species XF and XT



Specificity of Harper LAMP and Agdia kits

Label	Species / Description	Harper qPCR Ct Mean	Agdia- RPA	Agdia LFD	Harper LAMP	
					Tp	AD
DAR72015	Xanthomonas sp.	UD	UD	UD	UD	60.1
DAR65801	Stenotrophomonas	UD	UD	UD	UD	62.4
DAR72045	Stenotrophomonas	UD	UD	UD	UD	60.0
DAR75512	Stenotrophomonas	UD	UD	UD	UD	62.4
DAR76132	Stenotrophomonas	UD	UD	UD	UD	62.7
DAR77232	Stenotrophomonas	UD	UD	UD	UD	60.1
DAR77233	Stenotrophomonas	UD	UD	UD	UD	62.9
DAR77234	Stenotrophomonas	UD	UD	UD	UD	60.0
DAR77236	Stenotrophomonas	UD	UD	UD	UD	60.0
DAR77237	Stenotrophomonas	UD	UD	UD	UD	60.1
VPRI41552	Xanthomonas campestris	UD	UD	UD	UD	60.1
DAR82645	Xanthomonas campestris pv phaseoli	UD	UD	UD	UD	60.1
DAR82580	Xanthomonas sp.	UD	UD	UD	UD	60.1
DAR82611	Xanthomonas oryzae	UD	UD	UD	UD	60.0
DAR82627	Xanthomonas campestris pv pruni	UD	UD	UD	UD	60.0
P03- 83	Xanthomonas alfalfae subsp citrumelo	UD	UD	UD	UD	60.1
DAR33337	Xanthomonas arboricola	UD	UD	UD	UD	62.4
DAR30526	Xanthomonas campestris pv vitians	UD	UD	UD	UD	62.1
DAR41379	Xanthomonas vasicola	UD	UD	UD	UD	60.0
DAR35705	Xanthomonas translucens pv translucens	UD	UD	UD	UD	60.0
DAR49849	Xanthomonas hortorum	UD	UD	UD	UD	60.0
DAR73877	Xanthomonas vesicatoria	UD	UD	UD	UD	62.7
DAR82711	Xanthomonas curcurbitae	UD	UD	UD	UD	62.9
1622 B Strain	Xanthomonas fuscans subsp aurantifolii	UD	UD	UD	UD	62.1
Xff	Xf	21.3	positive	positive	7.3	89.9
Xt	XT	UD	positive	positive	6.8	89.5
Ntc	Molecular water	UD	negative	negative	UD	60.1

New Zealand	Victoria	New South Wales	Queensland	Western Australia	Northern Territory	South Australia	Norfolk Island
<i>Rosmarinus sp.</i> (Rosemary)	<i>Alnus sp.</i>	<i>Citrus sinensis</i> (Orange)	<i>Citrus aurantifolia</i> (Tahitian lime)	<i>Citrus limon</i> (Lemon)	<i>Citrus limon</i> (Lemon)	<i>Citrus reticulata</i> (Mandarin)	<i>Prunus</i> (Peach)
<i>Prunus armeniaca</i> (Apricot)	<i>Canna sp.</i>	<i>Citrus limon</i> (Lemon)	<i>Rubus idaeus</i> (Raspberry)	<i>Citrus spp.</i> (Limes)	<i>Citrus aurantifolia</i> (Tahiti lime)	<i>Citrus sinensis</i> (Orange)	<i>Vitis sp</i> (Grape)
<i>Prunus domestica</i> (Plum)	<i>Euphorbia sp.</i>	<i>Citrus reticulata</i> (Mandarin)	<i>Citrus australasica</i> (Finger Lime)	<i>Citrus sinensis</i> (Orange)	<i>Fortunella sp</i> (Kumquat)	<i>Citrus reticulata</i> 'Shiranui' (Dekapon)	<i>Prunus persica</i> (Peach)
<i>Prunus persica</i> (Plum)	<i>Ginkgo sp.</i>		<i>Citrus hystrix</i> (Kaffir lime)	<i>Citrus aurantifolia</i> (Tahiti lime)			<i>Citrus limon</i> (Lemon)
<i>Vitis sp.</i> (Grapevine)	<i>Nandina sp.</i>		<i>Citrus reticulata</i> (Mandarin)	<i>Olea europaea</i> (Olive)			<i>Persea americana</i> (Avocado, Hass)
<i>Quercus sp.</i> (Oak)	<i>Quercus sp.</i>		Tahiti lime x mandarin	<i>Vaccinium sect</i>			<i>Vitis sp</i> (Grapevine grown from seed)
<i>Acer pseudoplatanus</i> (Sycamore maple)							<i>Macadamia integrifolia</i> (Macadamia)
<i>Liquidambar sp.</i> (Sweetgum)							<i>Prunus domestica</i> (Plum)
<i>Polygala myrtifolia</i> (Milkwort)							<i>Pyrus pyrifolia</i> (Pear)
<i>Olea europaea</i> (Olive)	<i>Portulaca sp.</i> (Portulaca)		<i>Macadamia integrifolia</i> (Macadamia)	<i>Fragaria x ananassa</i> (Strawberry)			<i>Vitis sp</i> (Grape)
<i>Platanus acerifolia</i> (London Plane)	<i>Pelargonium sp.</i> (Pelargonium)		<i>Citrus limon</i> (Lemon)	<i>Citrus x aurantiifolia</i> (Lime)			<i>Citrus reticulata</i> (Mandarin)
<i>Acer sp.</i> (Maple)	<i>Salvia Rosmarinus</i> (Rosemary)		<i>Citrus x aurantiifolia</i> (Lime)	<i>Prunus dulcis</i> (Almond)			<i>Prunus</i> (Stone fruit)
	<i>Lavandula sp</i> (Lavender)		<i>Mangifera indica</i> (Mango)	<i>Nerium oleander</i> (Oleander)			<i>Citrus limon</i> (Breadfruit lemon)
	<i>Vitis sp</i> Grapevine		<i>Litchi chinensis</i> (Lychee)	<i>Citrus reticulata</i> (Mandarin)			<i>Moraceae</i> (Fig)
	Citrus spp.		<i>Olea europaea</i> (Olive)	<i>Macadamia integrifolia</i> (Macadamia)			<i>Citrus x sinensis</i> (orange)
			<i>Citrus maxima</i> (Pomelo)	<i>Quercus</i> (Oak)			<i>Nicotiana tabacum</i> (Tobacco)
			<i>Murraya koenigii</i> (Curry leaves)	<i>Acer</i> (Maple)			<i>Malus domestica</i> (Apple)
			<i>Eugenia uniflora</i> (Brazilian cherry)	<i>Citrus x paradisi</i>			<i>Prunus salicina</i>

Total of 403 plant samples were tested that covered 49 genera from AUS and NZ

Sampling

- Xylella is confined to the xylem tissue of its hosts
- Midribs, petiole, avoid fresh shoots, small plants whole leaves can be used.



DNA extraction-LAMP

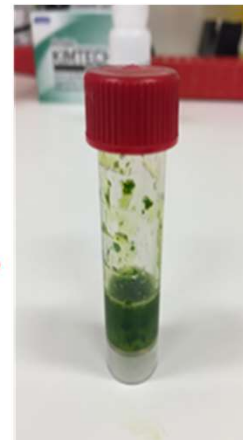
- DNA Extraction- in-field exaction kits -*Plant Lysis kit (Optigene)*



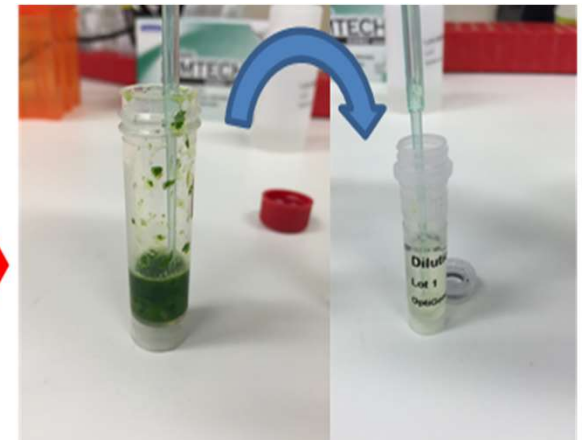
Plant lysis kit components



Step1- plant tissue and lysis buffer



Step 2 – Shake by hand to crush plant tissue



Step 3- Use the 10 ul loop to transfer homogenised tissue extract to dilution buffer- to be tested

How to determine DNA extraction worked?

Cox gene internal control

* Cox housekeeping LAMP (Tomlinson et al. 2012)

- ✓ Always include a negative and a positive control for each run
- ✓ negative control – Should be clear with no amplification
- ✓ A positive control – Plant DNA extracted from other method (DNeasy Kit) and you have tested it before

Loop- mediated Isothermal Amplification (LAMP)
requires 6 primers

LAMP test for Cox	Primer	Stock concentration (μM)	Amount to add for primer mix (uL)	Conc. in primer mix (uM)	Final conc. In reaction (uM)
Ratios			1:10:5		
* Cox house keeper	B3	10 μM	10	1	0.2 μM
	F3	10 μM	10	1	0.2 μM
	BIP	100 μM	10	10	2 μM
	FIP	100 μM	10	10	2 μM
	LF	100 μM	5	5	1 μM
	BF	100 μM	5	5	1 μM
Sub total			50		
H2O			50		
Total			100		

How to determine LAMP assay worked for *Xylella*?

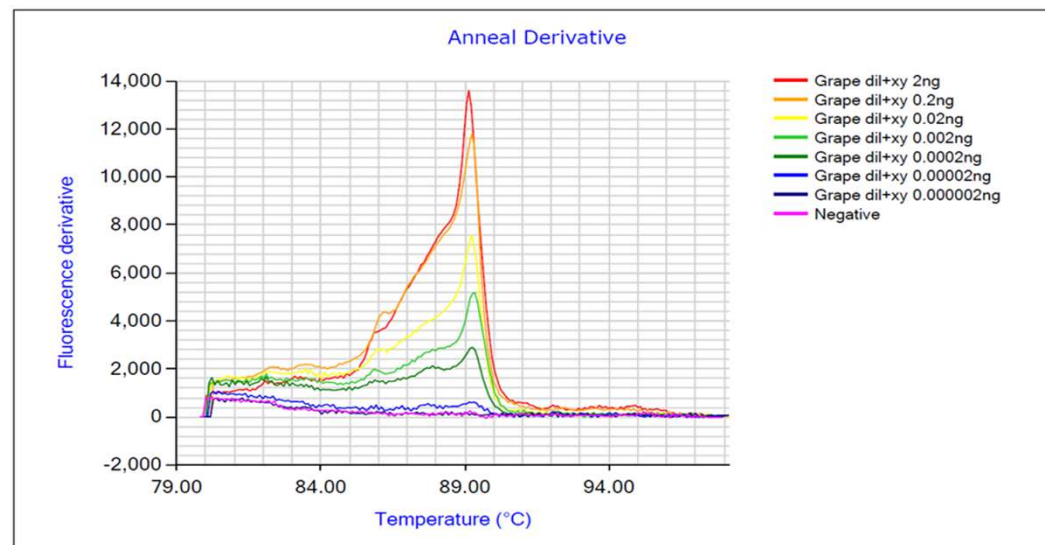
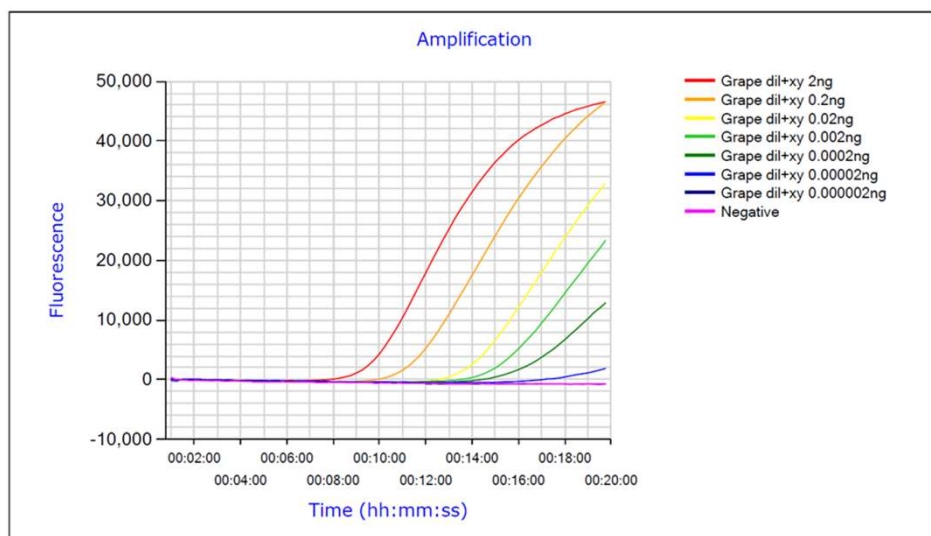
rim gene, LAMP of Harper et al (2010)

Detects all subspecies of *Xylella fastidiosa* and *Xylella taiwanensis*

- ✓ Positive controls *Xylella* DNA- ideally DNA extracted from colonies
- ✓ Positive control should have been tested in a qPCR, correlate Ct values to the Time to positive in LAMP
- ✓ There are two master mixes for LAMP by Optigen
 - DR001- Run time 30 min
 - DR004- Run time 20 min, its more robust

LAMP test for <i>X. fastidiosa</i>	Primer	Stock concentration	Amount to add for primer mix (uL)	Conc. in primer mix (uM)	Final conc. In reaction (uM)
<u>Ratios</u>			1:8:4		
<u><i>X. fastidiosa</i>-specific LAMP (Harper et al. 2010, erratum 2013)</u>			10	1	
	B3	10 µM			0.2 µM
	F3	10 µM	10	1	0.2 µM
	BIP	100 µM	8	8	1.6 µM
	FIP	100 µM	8	8	1.6 µM
	LF	100 µM	4	4	0.8 µM
	BF	100 µM	4	4	0.8 µM
<u>Sub total</u>			44		
<u>H2O</u>			56		
<u>Total</u>			100		

Interpretation of results



Well Number	Well Name(°C)	Peak Value(°C)
1	Grape dil+xy 2ng	89.1
2	Grape dil+xy 0.2ng	89.2
3	Grape dil+xy 0.02ng	89.2
4	Grape dil+xy 0.002ng	89.3
5	Grape dil+xy 0.0002ng	
6	Grape dil+xy 0.00002ng	
7	Grape dil+xy 0.000002ng	
8	Negative	

Recombinase polymerase RPA: Agdia kits

Local distributor

Email Susan.Archer@dpipwe.tas.gov.au

Department of Primary Industries, Parks, Water & Environment

BIOSECURITY TASMANIA

New Town Laboratories 13 St John's Avenue New Town, Tasmania, 7008

Ph. (03) 61653243

Web www.dpipwe.tas.gov.au

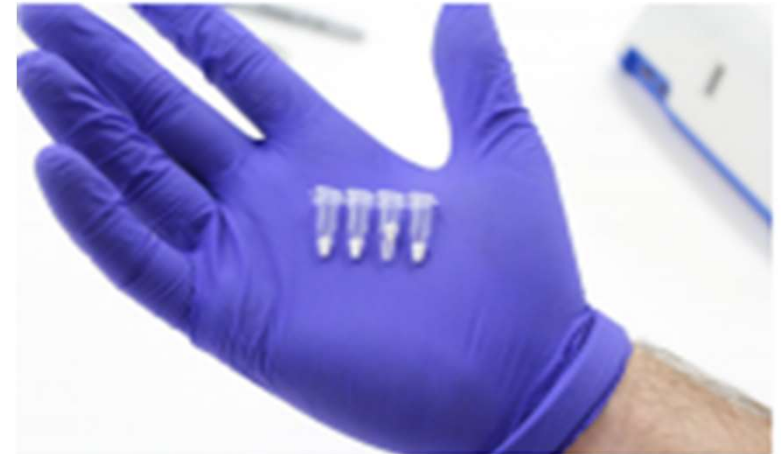
Agdia kits for the field day are in-kind provided by the manufactures- thanks to Susan Archer

Agdia Amplify RP® XRT kit for *Xylella fastidiosa*

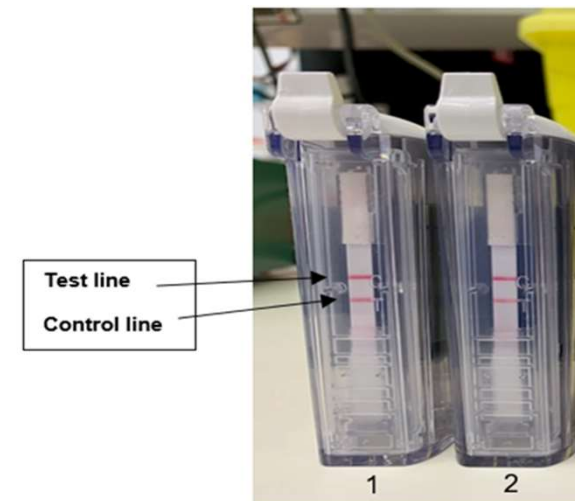
PART -1 XCS 34501/0048 kit contains following items

- XRT+ reaction pellets for Xf (48 individual tubes)
- PD1 Pellet diluent (55 mL)
- Amp1 extraction buffer (3 x 55 mL)
- Micro centrifuge tubes (50)
- Mesh extraction bags (50)

Part-2 AmplifyRP Amplicon Detection Chambers for endpoint detection



Lyophilized reaction mix



DNA extraction and RPA

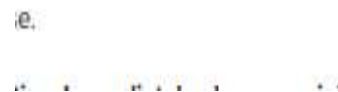


AmplifyRP® XRT+ for Xf
Rapid DNA Amplification Test Kit
Product No. XCS 34503

AmplifyRP® XRT+



1. Tissue from symptomatic almond leaf



Amplification at 42 C for 30 mins

Detection in Chambers

- ✓ Most field ready and transferable to field officers
- ✓ Less chances of contamination or cross- reaction
- ✓ Lyophilized reactions are stable
- ✓ Validated by AgVic

Interpret Results

Result	Lateral Flow Strip Reaction
Positive	Control and Test lines are both visible.
Negative	Control line is visible. Test line not visible.
Invalid	Control line not visible.



Step 1.



Step 2 (a).



Step 2 (b).



Step 3.



Step 4.



Acknowledgements

Susan Archer
Lucy Tran-Nguyen
Maggie Mwathi-Nyarko
Rachel Mann
Fiona Constable
Brandan Rodoni

Project partners MT17006- Improving preparedness of the Australian horticultural sector to the threat potentially posed by *Xylella fastidiosa* (a severe biosecurity risk)



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*Implementation
through connections*



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How to be generic but specific?

A nanopore sequencing-compatible PCR/qPCR assay for detection and differentiation of *Xylella fastidiosa* and *Xylella taiwanensis*

Johanna Wong-Bajracharya, John Webster, Luciano Rigano, Pragma Kant, Anna Englezou, Fridtjof Snijders, Rebecca Roach, Cuiping Wang, Monica Kehoe, Rachel Mann, Fiona Constable, Toni Chapman



ADSW 2023
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Department of
Primary Industries

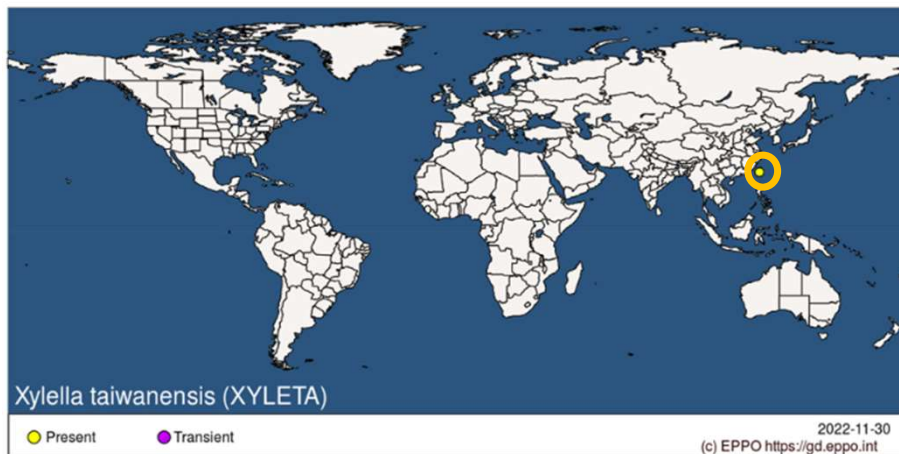
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Xylella taiwanensis is a lesser-known species in *Xylella* genus

Symptoms of pear leaf scorch



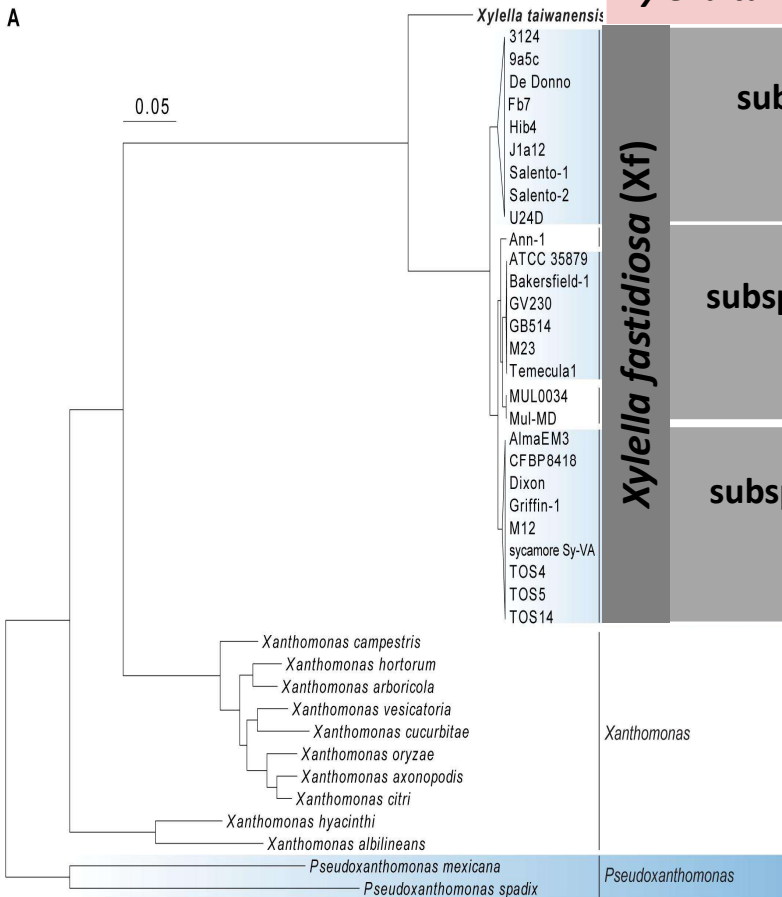
- Taiwan is the only region where *X. taiwanensis* is known to occur.
- Asian pear (*Pyrus pyrifolia*) is the only known host
 - **Potential host:** other *Pyrus* species such as Chinese pears (*Pyrus ussuriensis*), common pears (*Pyrus communis*)
- Presume to be transmitted by insect vector or grafting, but vector species is currently unknown
 - **Potential vector:** sharpshooter, leafhopper, froghopper, spittlebugs etc.
- Similar to *X. fastidiosa*, *X. taiwanensis* is xylem-limited.
- Symptoms of disease occurs 10-17 months after inoculation. Latency duration is unknown.
- Complete genome is available (Weng et al., 2021. *Front. Microbiol.*)

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Xylella taiwanensis is a lesser-known species in *Xylella* genus

A



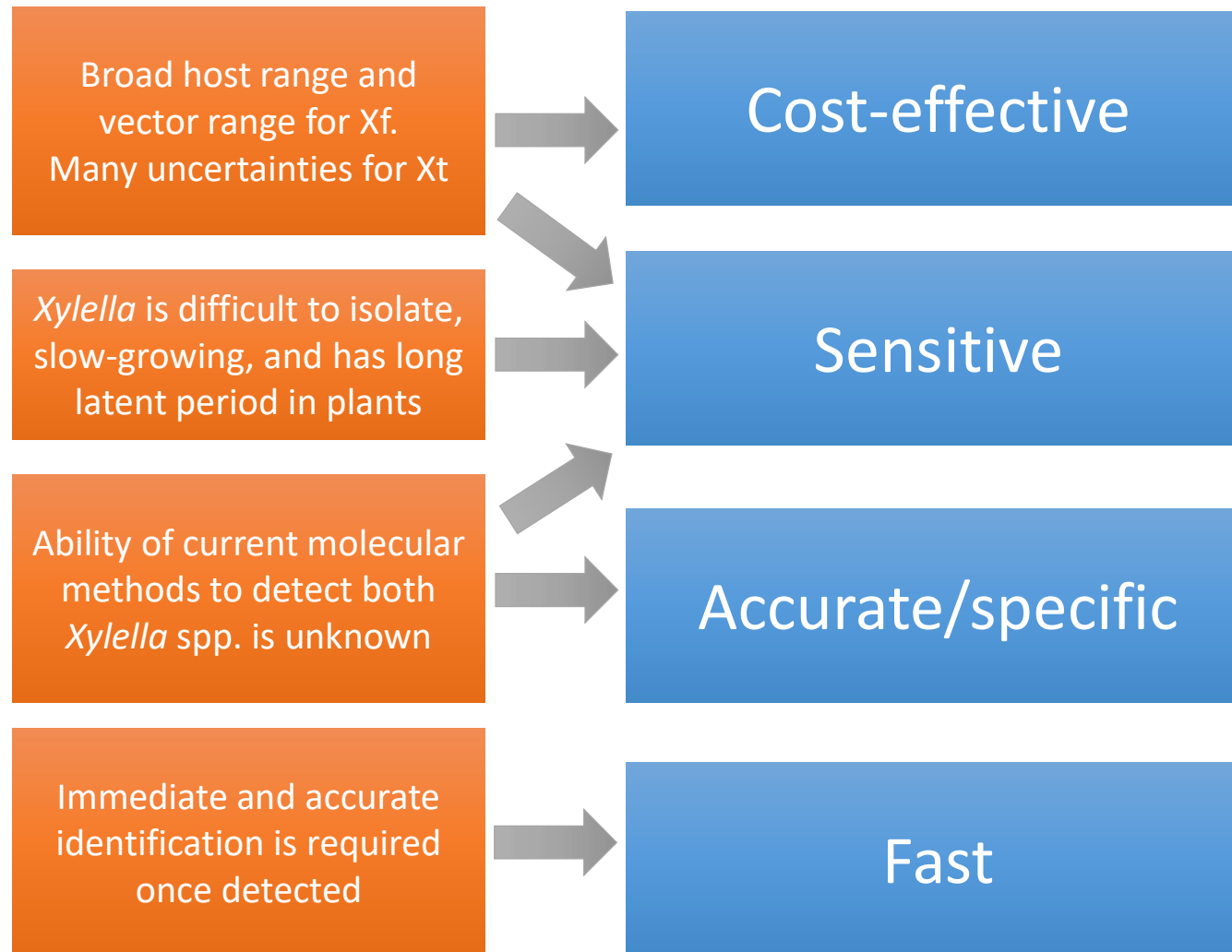
<i>Xylella taiwanensis</i> (Xt)		Pear leaf scorch	Asian pears	Taiwan only	2 PCR / qPCR assays
<i>Xylella fastidiosa</i> (Xf)	subsp. pauca (Xfp)	Citrus Variegated Cholorosis (CVC), Coffee leaf scorch	Citrus, coffee, olive etc.	Origin from America Severe damages in Europe, North and South America	<u>Traditional methods</u> • PCR / qPCR assays • MLST assays • LAMP assay • EPPO protocol <u>Next generation</u> • Targeted probe assay • Nanopore direct/amplicon sequencing
	subsp. fastidiosa (Xff)	Pierce's disease of grapevine	Grapevine, coffee, oleander etc.		
	subsp. multiplex (Xfm)	Plum leaf scorch, peach phony disease	Plum, peach, almond, olive etc.		
			> 650 species!	Ability in Xt detection is unknown!	

Xanthomonas

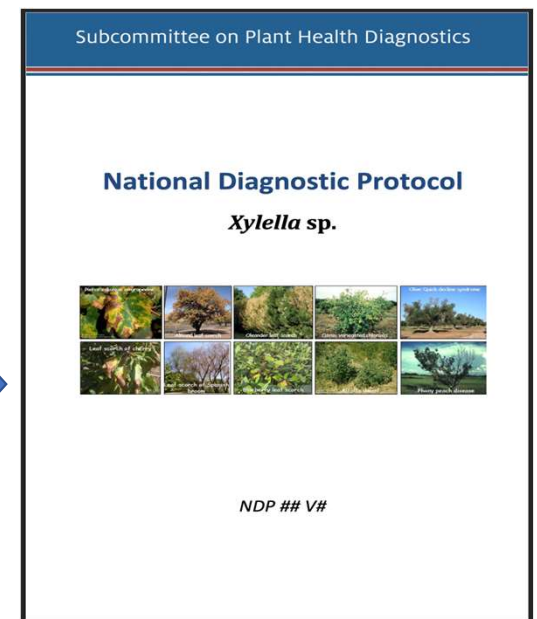
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Overcoming biosecurity challenges in *Xylella* detection



National Diagnostic Protocol development for Australia



Department of
Primary Industries and
Regional Development

Ministry for Primary Industries
Manatū Ahu Matua



QUEENSLAND
GOVERNMENT

Department of Agriculture and
Fisheries

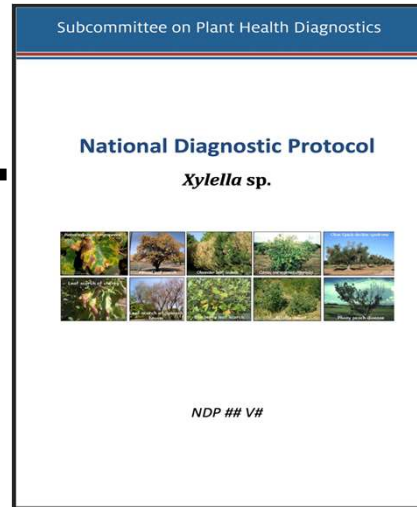
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National Diagnostic Protocol (NDP) development to safeguard Australia from *Xylella* spp.

Processing of samples &
isolation of bacterial cultures



Section 9 – surveillance samples

Generic *Xylella* Endpoint
PCR assay, with sequence
confirmation

Generic *Xylella* qPCR assay

Differentiation between
X. fastidiosa and *X. taiwanensis*

Molecular assays

Subspecies identification
multiplex qPCR

Genome sequencing

Multi-locus Sequence Typing
(MLST)

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Assessment of current molecular methods in detecting *Xylella* spp.

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Specificity testing

- 22 *Xylella* bacterial culture DNA
- 6 *Xylella* insect vector DNA
- 19 *Xylella*-infected plant DNA
- 25 *Xanthomonas/Stenotrophomonas* DNA

Sensitivity testing

- 4 representative *Xylella* bacterial culture DNA of different sp. and subsp.
- 14 serial dilution from 0.24 ng/μl

PCR methods:

- no/incorrect ID of Xt
- Mismatch to some Xf subsp. and no binding to Xt

	RST31/RST33 (Minsavage et al., 1994)	HL5/HL-ONT (Marcolungo et al., 2022)	X67S1/XL2r (Ito & Chiaki, 2021)
Xff	No mismatch	Target (CFBP8073) 3'-TTCCGTTATTTGTCGTGAT-5' 5'-AAGGCAATAAACGCGACTA-3' Forward primer (HL5) Target (CFBP8073) 3'-TTTGAGTCGGTAGCGCTT-5' 5'-AAACTCAGCCATTTCGCGAA-3' Reverse primer (HL-ONT)	No mismatch
Xfm	No mismatch	No mismatch	No mismatch
Xfp	Target (Salento-1) 3'-CGCAATTAAAGCTTCACTAAGCTAAAG-5' 5'-GCGTTAATTTTCGAAGTGATTGATTGC-3' Forward primer (RTS31)	No mismatch	Target (6c) 3'-CCTGCCGTCGTGCAACCAT-5' 5'-GGACGGCAGCACATTGGTA-3' Forward primer (X67S1)
Xt	No binding	No binding	Target (PLS229) 3'-CTGCGTCGTGTACCAT-5' 5'-GACGGCAGCACATTGGTA-3' Forward primer (X67S1)

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Assessment of current molecular methods in detecting *Xylella* spp.

Specificity testing

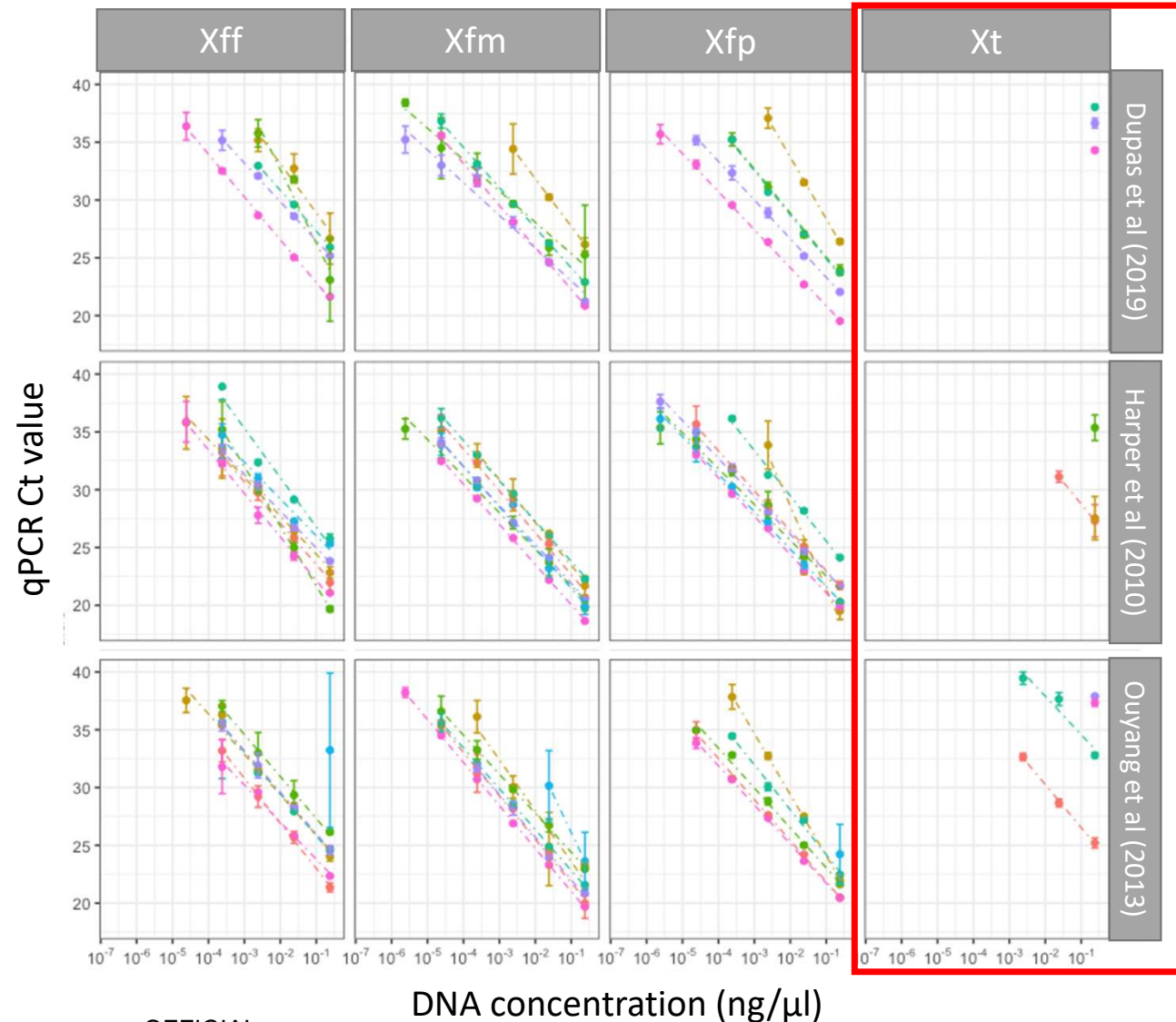
- 22 *Xylella* bacterial culture DNA
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Sensitivity testing

- 4 representative *Xylella* bacterial culture DNA of different sp. and subsp.
- 14 serial dilution from 0.24 ng/μl

qPCR methods:

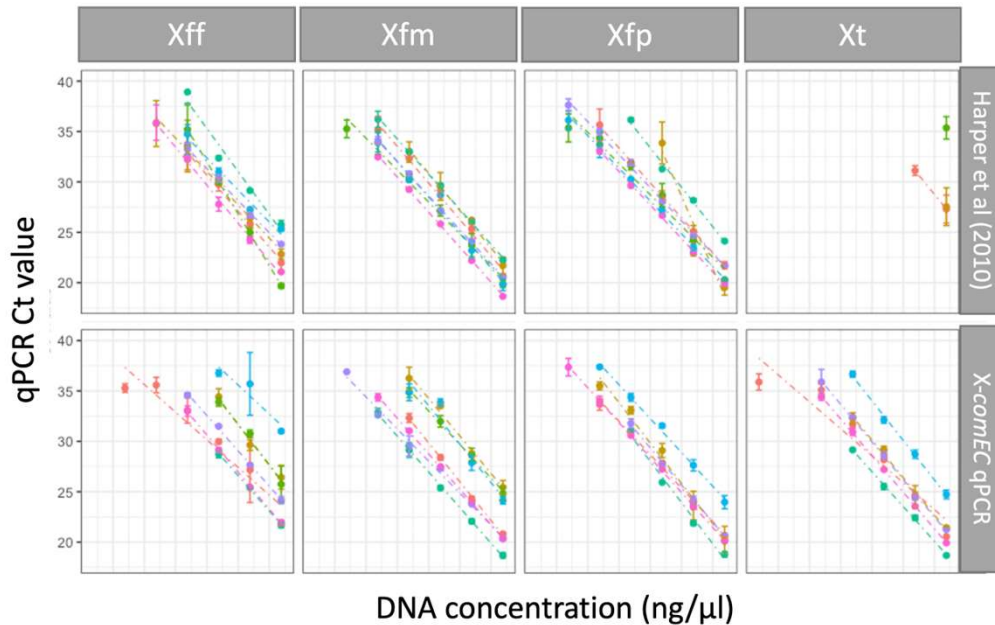
- Testing across 5 national diagnostic labs
- Detection limit = approx. 2.4×10^{-4} to 10^{-5} ng/μl across all Xf subsp.
- Xf qPCR described by Harper et al. (2010) is most consistent
- All tested methods could not detect Xt reliably



X-comEC PCR/qPCR can detect both *Xylella* spp.

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qPCR detect Xt with superior sensitivity



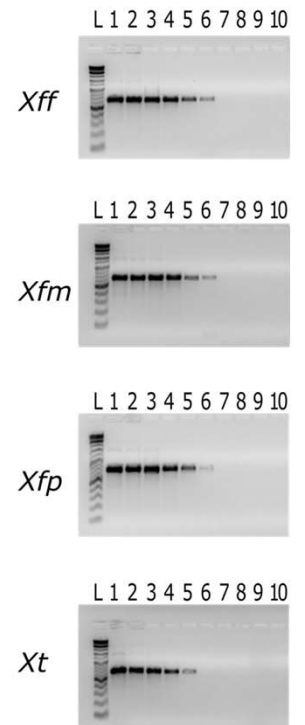
- Both PCR and qPCR assays are specific to *Xylella*
- No cross-reactivity to 161 plant samples of different species sampled across Aus/NZ

PCR performance consistent across 7 testers Detection limit (35 cycle) = 2.4×10^{-4} to 10^{-5} ng/μl

(a)

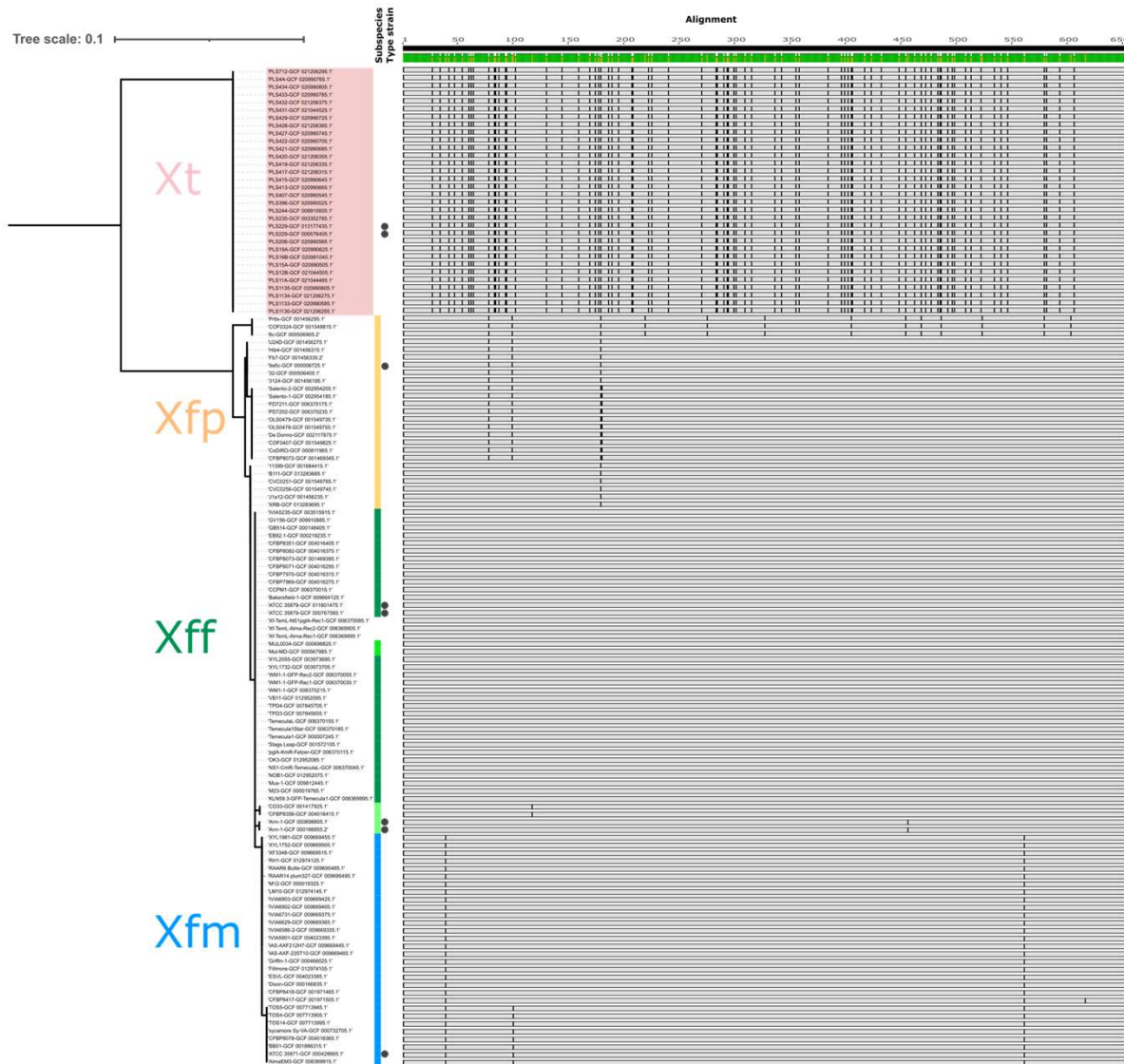
		Tester		A	B	C	D	E	F	G
		Reagent		MyTaq	MyTaq	MyTaq	GoTaq	Mango Taq	GoTaq	GoTaq
		PCR cycle		35	35	40	35	35	35	35
Sample	copies/μl	ng/μl	Result							
Xff	87540	2.40E-01								
	8754	2.40E-02								
	875.4	2.40E-03								
	87.54	2.40E-04								
	8.754	2.40E-05								
	0.8754	2.40E-06								
	0.08754	2.40E-07								
	0.008754	2.40E-08								
	0.0008754	2.40E-09								
	0.00008754	2.40E-10								
Xfm	87540	2.40E-01								
	8754	2.40E-02								
	875.4	2.40E-03								
	87.54	2.40E-04								
	8.754	2.40E-05								
	0.8754	2.40E-06								
	0.08754	2.40E-07								
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	0.00008754	2.40E-10								
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	875.4	2.40E-03								
	87.54	2.40E-04								
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	0.8754	2.40E-06								
	0.08754	2.40E-07								
	0.008754	2.40E-08								
	0.0008754	2.40E-09								
	0.00008754	2.40E-10								
Xt	87540	2.40E-01								
	8754	2.40E-02								
	875.4	2.40E-03								
	87.54	2.40E-04								
	8.754	2.40E-05								
	0.8754	2.40E-06								
	0.08754	2.40E-07								
	0.008754	2.40E-08								
	0.0008754	2.40E-09								
	0.00008754	2.40E-10								

(b)



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Differentiation of different *Xylella* lineages by X-comEC PCR



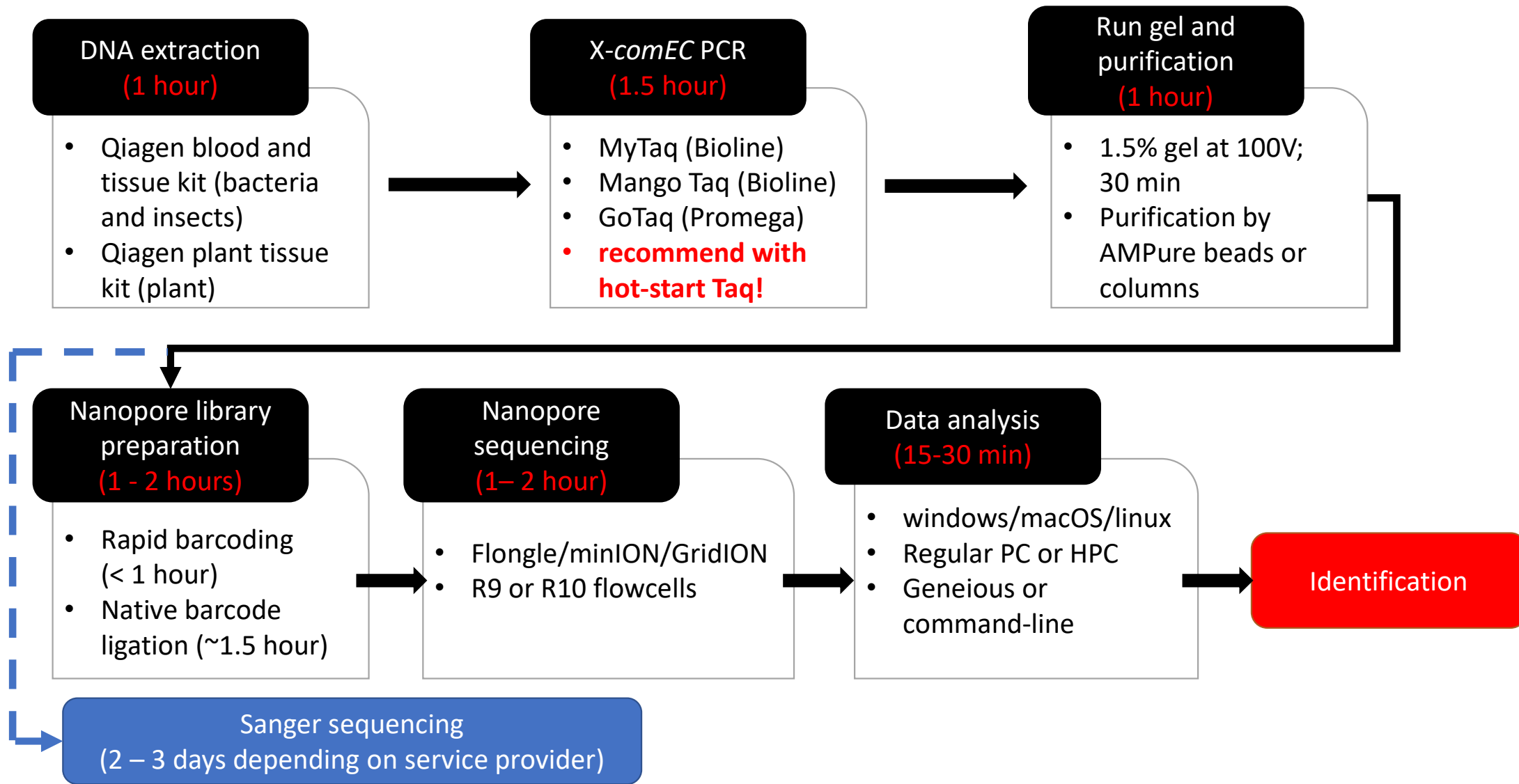
- X-comEC PCR amplicon extracted from 129 *Xylella* genomes
- Sequence variation according to species and subspecies differences
- Sequencing of X-comEC PCR amplicon can be used for *Xylella* identification at species or subspecies level
- Tested to work with Nanopore sequencing device



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X-comEC PCR procedure in NDP: from DNA to identification

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Different consensus-calling methods could yield varying results

OFFICIAL

- Correct ID to almost all positive samples

				NGSpeciesID			medaka			Geneious		
Sample ID	Read count	N50	True identity	Closest match	Identity (%)	Query coverage (%)	Closest match	Identity (%)	Query coverage (%)	Closest match	Identity (%)	Query coverage (%)
ICMP8731	36,345	412	Xff	Xff	98.92	85.54	Xff	100	99.38	Xff	100	96.62
ICMP8739	24,106	417	Xfm	Xfm	100	96.15	Xfm	100	99.38	Xfm	100	97.38
ICMP8740	24,316	409	Xfm	Xfm	99.18	93.69	Xfm	100	98.77	Xfm	100	96.62
ICMP8745	24,622	414	Xff	Xff	99.16	91.69	Xff	100	99.38	Xff	100	97.54
NCPB4432	33,901	412	Xff	Xff	99.02	93.85	Xff	100	100	Xff	100	96.92
CFBP8495	48,794	414	Xfp	Xfp	98.45	84.46	Xfp	100	99.08	Xfp	100	96.92
CFBP8072	37,609	412	Xfp	Xfp	99.66	91.54	Xfp	100	99.85	Xfp	100	96.62
CFBP8077	23,183	413	Xff	Xff	100	95.08	Xff	100	99.38	Xff	100	96.92
NCPB4612	36,998	423	Xt	Xt	98.56	96	Xt	100	99.85	Xt	100	96.62
Xf vitis stock	91	120	Xff	no match	na	na	Xff	97.55	94.15	no match	na	na
DAR77232	3,459	191	Stenotrophomonas	Xff*	98.94	58	Xff*	99.21	96.92	no match	na	na
Xf plant B2S4	21,572	416	Xff	Xff	99.52	96.77	Xff	100	99.54	Xff	100	96.92
Xf plant B3S1	14,780	417	Xff	Xff	99.84	95.23	Xff	100	99.85	Xff	100	96.92
Xf plant B4S2	10,905	423	Xff	Xff	99.83	90.31	Xff	100	99.38	Xff	100	97.08
Xf plant B5S1	28,187	418	Xff	Xff	98.83	92.31	Xff	100	99.08	Xff	100	96.62
Xf exposed beetles	17,612	424	Xfm	Xfm	99.53	97.08	Xfm	100	99.23	Xfm	100	96.92
No template	864	233	0.69	no match	na	na	Xfm*	95.69	78.46	no match	na	na

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Different consensus-calling methods could yield varying results

OFFICIAL

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- Trouble in low read count sample

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No template	864	233	0.69	no match	na	na	Xfm*	95.69	78.46	no match	na	na

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Different consensus-calling methods could yield varying results

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- Correct ID to almost all positive samples
- Trouble in low read count sample
- Medaka method gives false-positive ID to non-Xylella and no template sample!
- Therefore, medaka is not recommended

Sample ID	Read count	N50	True identity	NGSpeciesID			medaka			Geneious		
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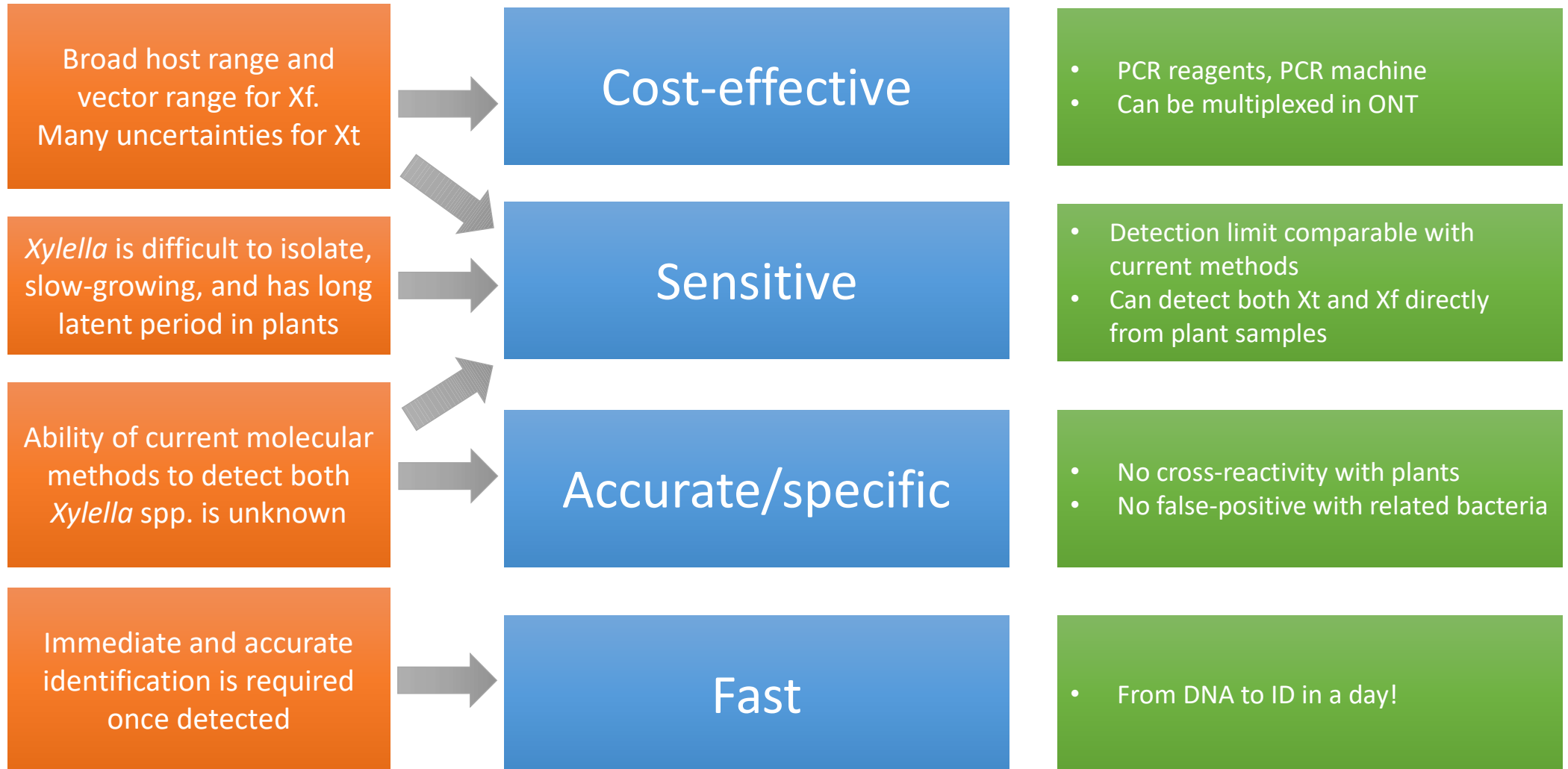
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Overcoming biosecurity challenges in *Xylella* detection

X-comEC PCR



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OFFICIAL Acknowledgement

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Article title

High-quality full genome assembly of historic *Xylella fastidiosa* strains from ICMP collection using a hybrid sequencing approach

Authors

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Running title

Hybrid assemblies of *Xylella fastidiosa*

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[#]Present address Rachel Mann: Biosecurity and Agriculture Services, Attwood, Victoria, Australia.

Keywords: Bacteria, long and short reads, MinION, Nanopore, Subspecies.

Abstract

High-quality complete genomes of five *Xylella fastidiosa* strains (ICMP 8731, ICMP 8739, ICMP 8740, ICMP 8742 and ICMP 8745) were assembled by combining Nanopore and Illumina sequencing data. Cultures were originally from USA and obtained from International Collection of Micro-organisms from Plants (ICMP), Manaaki Whenua – Landcare Research, Auckland, New Zealand.

Announcement

Xylella fastidiosa is a xylem-limited plant pathogenic bacterium that has caused significant epidemics and economic losses in many plant species in the Americas and Europe. This bacterium is the number one quarantine pathogen for Australia and New Zealand. The genus *Xylella* has complex taxonomy, having two species: *fastidiosa* and *taiwanensis*. Within the *fastidiosa*, there are six known subspecies, three of them, *fastidiosa*, *multiplex* and *pauca*, are of significance based on the genomic analysis. The other three subspecies, *sandyi*, *taske* and *morus* are part of the larger group *fastidiosa sensu largo* (Denancé et al 2019).

X. fastidiosa strains, originally isolated in the USA in 1980s, were collected by ICMP, New Zealand. The strain ICMP 8731 was isolated from *Vitis vinifera* and ICMP 8745 from *Ambrosia*, respectively, in Florida. The strains ICMP 8740 and ICMP 8742 were collected from host *Platanus occidentalis* and *Ulmus americana*, respectively, in Washington, DC. and ICMP 8739 was collected from *Prunus dulcis* in California. High purity DNA from all *X. fastidiosa* strains was isolated from a month-old single cell colonies grown on BYCE modified media at 25°C (Wells et al. 1981) by using a Blood and Tissue DNeasy kit (Qiagen) according to the manufacturer's instructions.

Illumina sequencing libraries were generated using PerkinElmer NEXTFLEX Rapid XP DNA-Seq Kit at half reaction with PerkinElmer NEXTFLEX® UDI Barcodes (PerkinElmer, Massachusetts, United States). Libraries were paired end sequenced (2 × 150 bp) using the Illumina NovaSeq 600. For Nanopore Sequencing, 400 ng DNA of each strain was used in library preparation with Rapid barcoding kit RBK004 following manufacturer's instructions. The final library was loaded onto a R9.4 Flowcell in a MinION Mk1B device (Oxford Nanopore Technologies). The sequencing was controlled with MinkNOW software version 22.11.7. for 24 hrs generating 6 Gb of data. Output fast5 files were processed using super accuracy base calling model for fastq files.

Hybrid assemblies were performed by Unicycler version 0.4.7 using both Nanopore and Illumina reads.

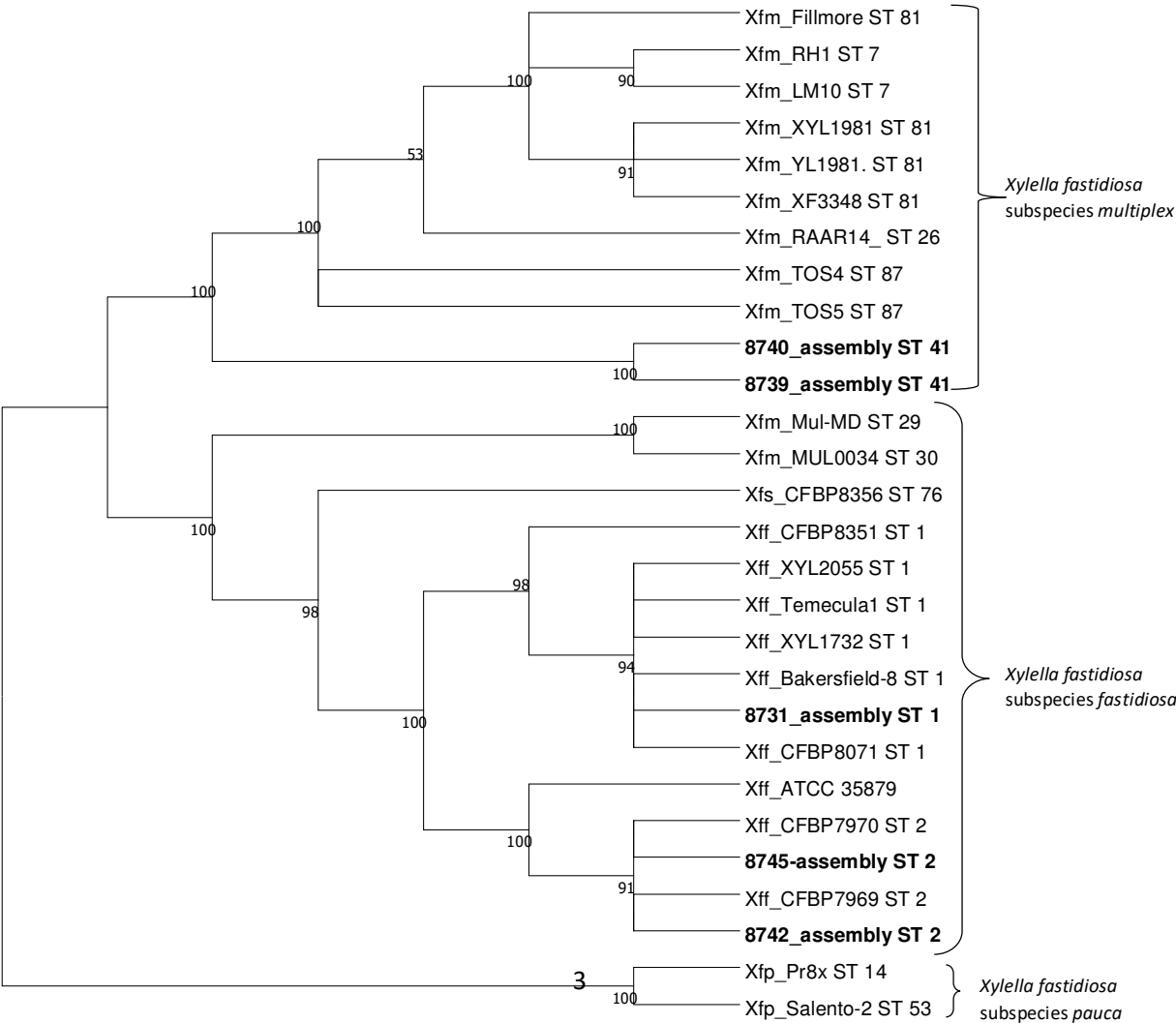
All *X. fastidiosa* strains were circularised in a chromosome except for ICMP 8739 and ICMP 8745, that were assembled with gaps and nodes at the end, respectively. Annotations were generated by NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) pipeline (Tatusova et al. 2016) (Table 1). Both the genome sizes and GC content were comparable with *X. fastidiosa* reference genome (GCA_011801475.1) available in NCBI (2.5Mb and 51.5% - 52.4% respectively).

Table 1: Description and annotation metrics of the five *Xylella fastidiosa* strains hybrid assemblies generated in this work.

Strain name	ICMP 8731	ICMP 8739	ICMP 8740	ICMP 8742	ICMP 8745
Subspecies	fastidiosa	multiplex	multiplex	fastidiosa	fastidiosa
LocusTagPrefix	PT013	PRA06	PT012	PUO95	PRA13
BioSample	SAMN33037963	SAMN33037965	SAMN33037964	SAMN33037966	SAMN33037967

Genbank accession	CP117821	JAQQT01000000	CP117820	CP117823	JAQQT000000000
Genomesize (bp)	2409646	2023822	2548838	2556755	2589186
CDS	2084	1711	2227	2252	2308
Gene	2136	1765	2286	2311	2367
ncRNA	3	3	3	3	3
Regulatory	1	1	1	1	1
rRNA	3	6	6	6	6
tmRNA	1	1	1	1	1
tRNA	45	44	49	49	49
Plasmid name	pXFAS01			pXF879-41	
Plasmid size (bp)	38298			41752	

Assembled genomes were used to determine sequence types using linux based single script <https://github.com/tseemann/mlst>. Strain ICMP 8731 was identified as ST 1 and ICMP 8742, ICMP 8745 were identified as ST 2. ICMP 8739 and ICMP 8740 were identified as ST 41. A maximum-likelihood phylogenetic tree using RAxML version 8.2.8 (Stamatakis, 2014) was generated with UBCG, a core genome analysis pipeline (Na et al. 2018) that extracts and concatenates 92 core bacterial genes. All six strains were placed correctly to the respective clades in the dendrogram (Figure 1).



71

72 Figure 1. The phylogenetic tree showing the grouping of the 6 isolates inferred using the Neighbor-joining method
 73 (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.03980352 is shown. The tree is drawn to
 74 scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic
 75 tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and
 76 Kumar 2004). The analysis involved 29 genomes sequences, sequence types indicated as ST and names in bold are
 77 from this study. Evolutionary analyses were conducted in MEGA7 (Kumar et al.2016). CFBP, French Collection for
 78 Plant Associated Bacteria; ATCC, American Type Culture Collection, USA; ICMP, International Collection of
 79 Microorganisms from Plants, New Zealand.

80 Data availability

81 Genome assemblies are available from GenBank database as Bioproject- PRJNA930964 having five accessions
 82 CP117821, JAQQTM01000000, CP117820, CP117823, JAQQTL000000000.

83 Acknowledgments

84 This study was undertaken as part of the project MT17006 Improving preparedness of the Australian horticultural
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 86 Frontiers Advanced Production Systems fund, part of the Hort Frontiers strategic partnership initiative developed
 87 by Hort Innovation, with co-investment from Department of Jobs, Precincts and Regions Victoria, Department of
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 89 Fisheries, Queensland and Department of Primary Industries and Regional Development Western Australia and
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91 References

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 93 subspecies signatures in *Xylella fastidiosa*. BMC genomics, 20(1): 1-21.
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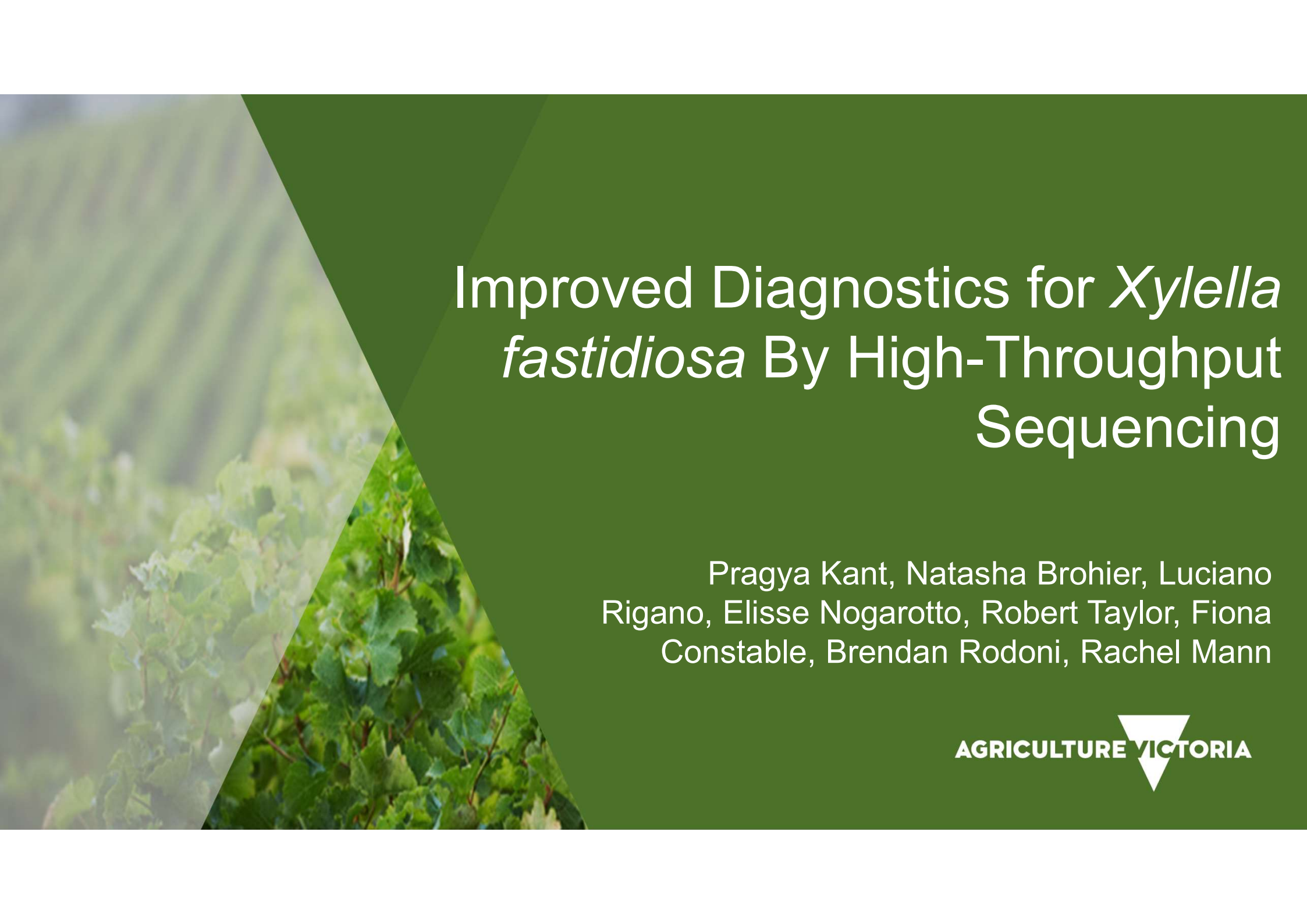
Improved Diagnostics for *Xylella fastidiosa* By High- Throughput Sequencing

Pragya Kant

Research Scientist

Agriculture Victoria Research, Department of Jobs, Precincts and Regions



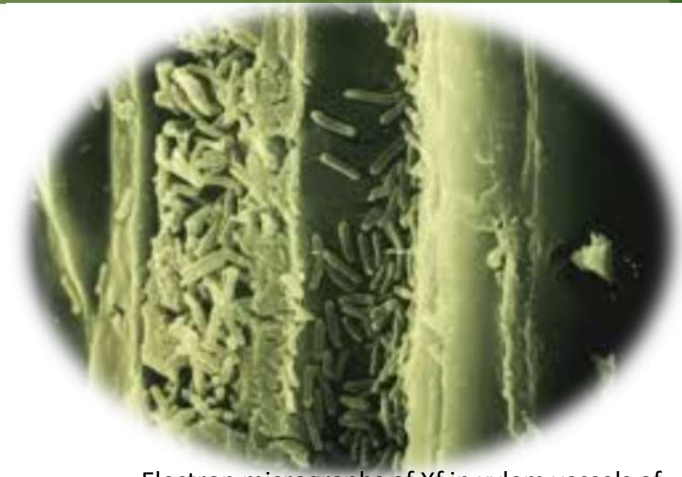


Improved Diagnostics for *Xylella fastidiosa* By High-Throughput Sequencing

Pragya Kant, Natasha Brohier, Luciano Rigano, Elisse Nogarotto, Robert Taylor, Fiona Constable, Brendan Rodoni, Rachel Mann

Background

- Gram-negative bacterium
 - Xylem limited in plants
 - Transmitted by Hemiptera species
 - Sharpshooter, leafhoppers
 - spittlebugs
-
- ✓ Xylella not present in Australia and New Zealand
 - ✓ Xylella is #1 on Australia's National Priority Plant Pest list
 - ✓ Huge economic losses



Electron micrographs of Xf in xylem vessels of grapevine © Dr. Doug Cook UC Davis

Protecting Australian plants from Xylella

Learn to identify Xylella and exotic insect vectors

Symptoms of the disease include leaf scorching and browning, loss of leaves, gradual decline in fruit size, stunted growth, dieback and, eventually, plant death.

The bacteria can be spread by many plant-sucking insects such as spittle bugs and the exotic **glassy winged sharpshooter** (*Homalodisca vitripennis*). Glassy winged sharpshooters are large insects (about 12-14 mm long). They are dark brown with stippled spots on



Image: John Hartman, University of Kentucky, Bugwood.org

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Image: John Hartman, University of Kentucky, Bugwood.org

Symptoms



Blueberry



Almond



Pecan



Grapevine



Avocado



Oleander



Citrus

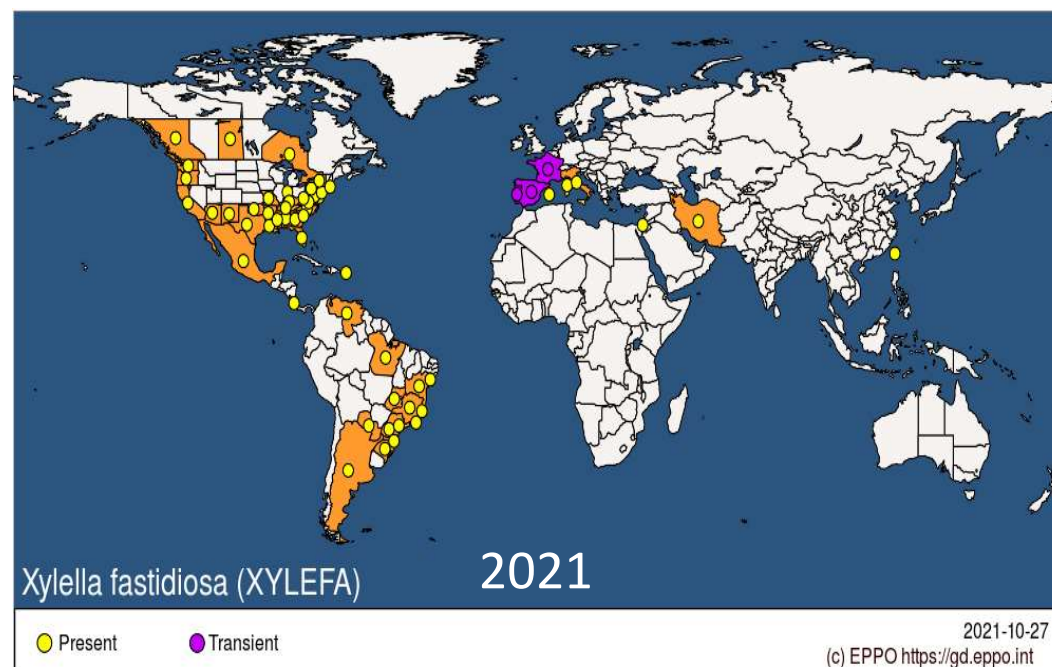


Olives

Expanding Host Range and Distribution

	2016	2018	2020
Plant species reported infected	359	563	638
Genera	204	264	289
Botanical families	75	82	87

EFSA Journal 2016;14(2):4378
 EFSA Journal 2018;16(9):5408
 EFSA Journal 2021;19(6):6674



- Currently in the Americas, Spain, Italy, France, Iran, Israel and Taiwan
- X. fastidiosa* may not cause disease in all species
 - May be associated with lower level of colonisation
 - Threat to native species

Xylella Taxonomy

Two species: *Xylella fastidiosa* and *Xylella taiwanensis*

Subspecies: six different subspecies of *X. fastidiosa* have been proposed

- *X. fastidiosa* subsp. *fastidiosa*
- *X. fastidiosa* subsp. *multiplex*
- *X. fastidiosa* subsp. *pauca*
- *X. fastidiosa* subsp. *sandyi*
- *X. fastidiosa* subsp. *tashke*
- *X. fastidiosa* subsp. *morus*

sandyi & *morus* now considered part of *fastidiosa*, *tashke* is not isolated since identified

Further classified into Sequence types (ST)

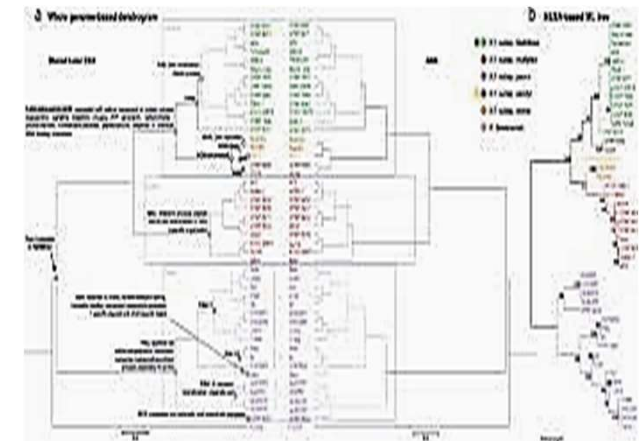
Sequence types are important to know- diagnostic gold !!

Importance of Sequence Typing

- Sequence types (STs): Multi-locus sequence typing- characterise *X. fastidiosa*
- *X. fastidiosa* MLST by Scally et al 2005
- 7 genes for *X. fastidiosa* *cys*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC*
- Different alleles for each gene- 149 alleles, 89 known STs (PubMLST db)
- STs are associated with host specificity and introduction
- Example - outbreaks in the EPPO region: *X. fastidiosa* subsp. *pauca* (ST53)
 - *Xf pauca* (main hosts: Citrus and Coffea spp.)
 - didn't cause citrus variegated chlorosis and coffee leaf scorch, this strain infected Olives in Italy

Traditional MLST

- Conducted on the culturable pathogens (months to culture *Xylella*)
- PCR and Sanger sequencing – days for results
- Limited understanding of evolution and inter sub-species recombination



High-throughput Sequencing for Improved and Rapid Diagnostics

- ✓ Cheaper to do HTS
- ✓ Time saving
- ✓ Applied directly on host samples
- ✓ More data means more information
- ✓ Handle large number of samples

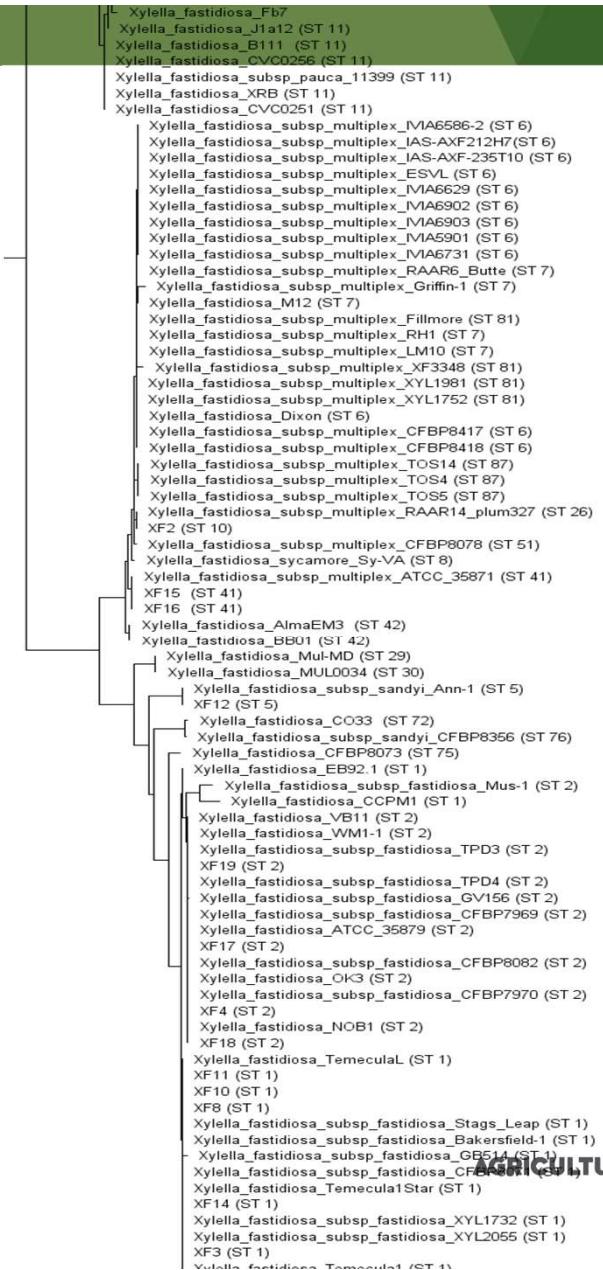
ST assignments using third-generation sequencing platforms:

- Illumina WGS, core genome to get ST (UBCG)
- Oxford Nanopore Technologies- amplicon MLST sequencing directly from plants
- Assign ST from metagenomic data (StringMLST, MetaMLST, MLST, PubMLST)



Up-to-date-Bacterial Core Genome

- Publicly available pipeline: UBCG- (up-to-date bacterial core gene set)- Na et al. 2018
- Uses 92 core genes- bacteria and archaea
- Implement as part of the standard HTS pipeline for bacterial ID in our lab
 - No need for individual genus-based databases e.g. MSLT/MLSA
 - No PCR bias
- 115 *Xylella* genomes- sequence and NCBI
 - Accurate subspecies differentiation
 - ST clustering



Sequencing of Metagenomic Samples

Illumina Miseq

Spiked Concentration of Xylella DNA		qPCR (Ct)	LAMP (amp time)	StringMLST			Total Xylella contig			Genome coverage (%)		
				2 GB	5 GB	10 GB	2 GB	5 GB	10 GB	2 GB	5 GB	10 GB
No dilution	0.24 ng/μl	21.09	9.9	ST2	ST2	ST2	2561	1486	318	77.5	93.62	99.67
10 ⁻¹	24 pg/μl	24.52	11.2	XF	XF	ST2	133	488	1233	1.84	7.23	20.59
10 ⁻²	2.4 pg/μl	27.87	12.9	UD	UD	XF	3	11	45	0.05	0.142	0.619
10 ⁻³	240 fg/μl	31.17	14.7	UD	UD	UD	1	1	1	0.012	0.011	0.013
10 ⁻⁴	24 fg/μl	34.21	20.4	UD	UD	UD	UD	UD	UD	UD	UD	UD
10 ⁻⁵	2.4 fg/μl	37.45	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
10 ⁻⁶	240 ag/μl	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
10 ⁻⁷	24 ag/μl	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

- qPCR, limited identification at species, sub-species and ST levels
- UD- undetermined

Amplicon Sequencing- MLST genes

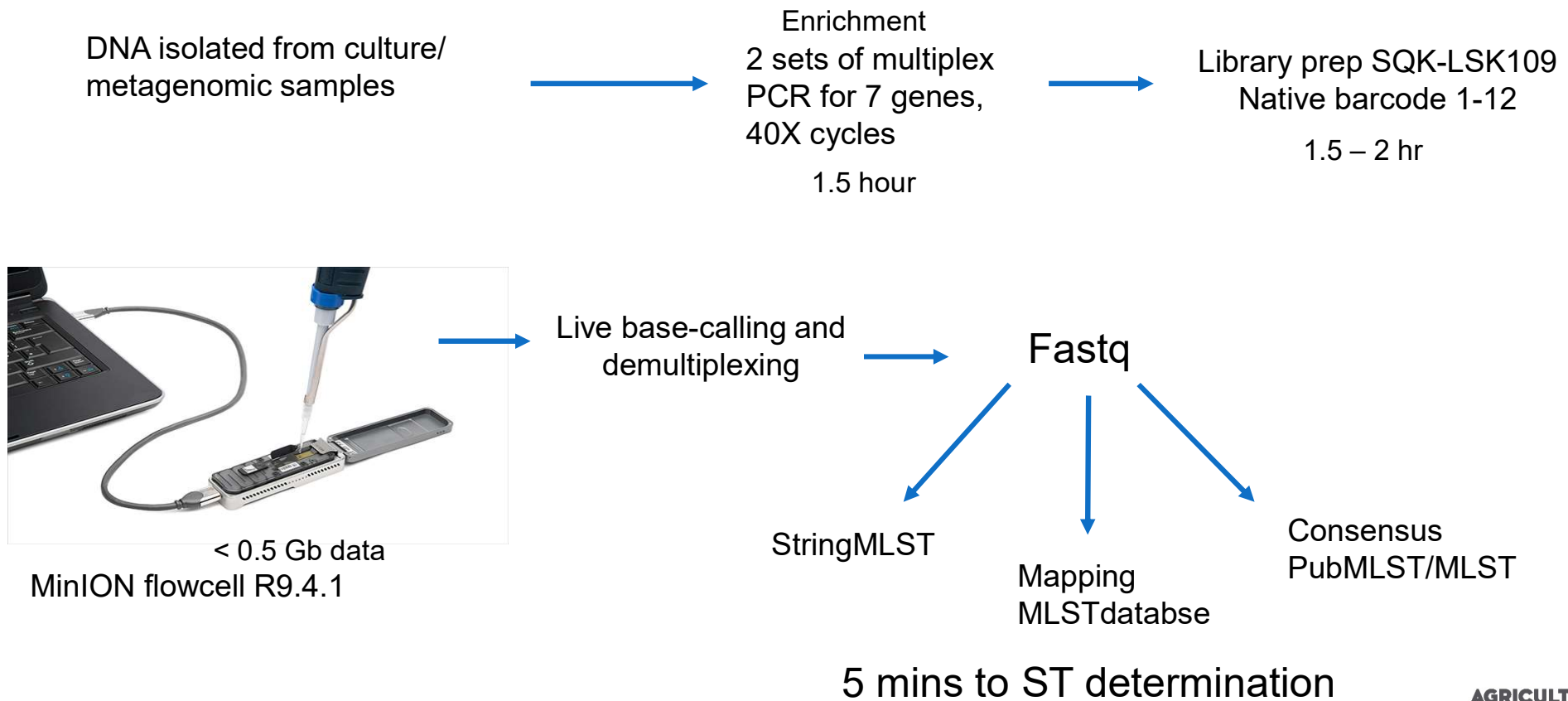
- Metagenomic samples
- Targeted approach
- Enrichment with PCR 40X cycles - Faino et al 2019
- Oxford Nanopore MinION- allows portability

leuA, petC, malF, cysG, holC, nuoL, gltT - NDP

Advantages

- Transferrable from lab to lab
- Can detect any mixing of the isolates
- Allelic profile and introductions
- High-throughput platform for large-scale pathogen typing

A day MinION workflow to determine ST



Higher Sensitivity of ONT-MLST

Spiked concentration of Xf DNA		qPCR assays		LAMP assays	ONT-MLST amplicon seq
		Haper et al 2010	Dupas et al 2019 (generic)	Harper et al 2010 (Tp)	
No dilution	240 pg/μl	21.90	26.38	9.9	Not tested
10 ⁻¹	24 pg/μl	24.52	29.46	11.2	Not tested
10 ⁻²	2.4 pg/μl	27.87	33.17	12.9	ST
10 ⁻³	240 fg/μl	31.17	36.16	14.7	ST
10 ⁻⁴	24 fg/μl	34.21	39.00	20.4	ST
10 ⁻⁵	2.4 fg/μl	37.45	UD	UD	ST
10 ⁻⁶	240 ag/μl	UD	UD	UD	ST
10 ⁻⁷	24 ag/μl	UD	UD	UD	XF
10 ⁻⁸	2.4 ag/μl	UD	UD	UD	UD

Take Away Messages

- High-throughput sequencing has revolutionised diagnostics
- ONT-MLST is most sensitive and fastest approach for determination of ST
 - 100 fold more sensitive to qPCR (ST determination)
 - 1000 fold more sensitivity to qPCR (species identification)
 - Accurate, high-throughput platform for large scale pathogen typing
 - Portable with potential to work in-field
- *Xylella fastidiosa* is #1 National priority plant pest. *Xylella* is off-shore and want to keep it that way...**never ignore Australia's strict biosecurity rules**.....do your part!!

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Acknowledgements

Project: Improving preparedness of the Australian horticultural sector to the threat potentially posed by *Xylella fastidiosa* (a severe biosecurity risk)

**Horticulture
Innovation
Australia**



Queensland Government
Department of Agriculture and Fisheries



Department of
**Primary Industries and
Regional Development**



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Primary Industries**



Ministry for Primary Industries
Manatū Ahu Matua



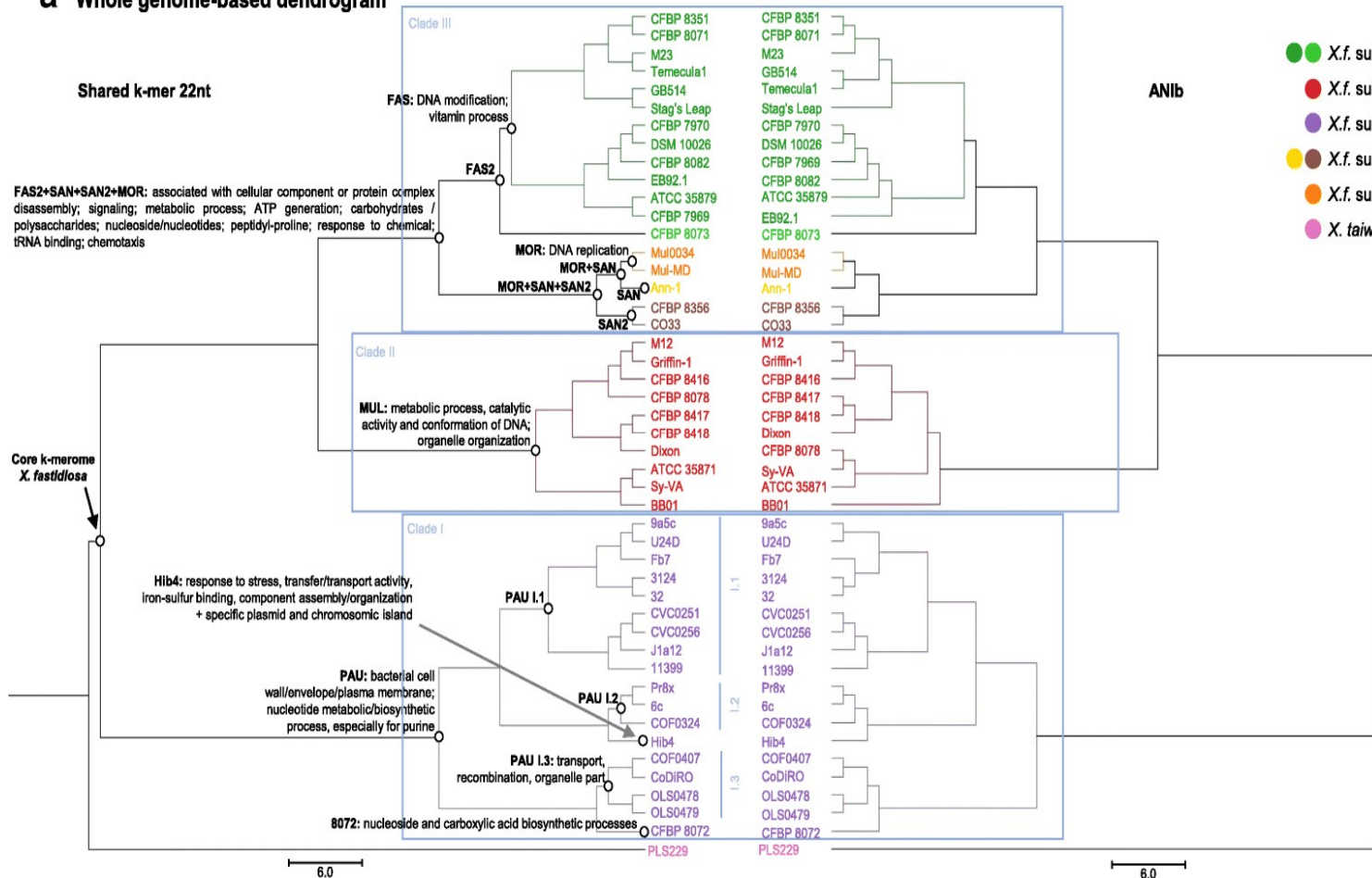
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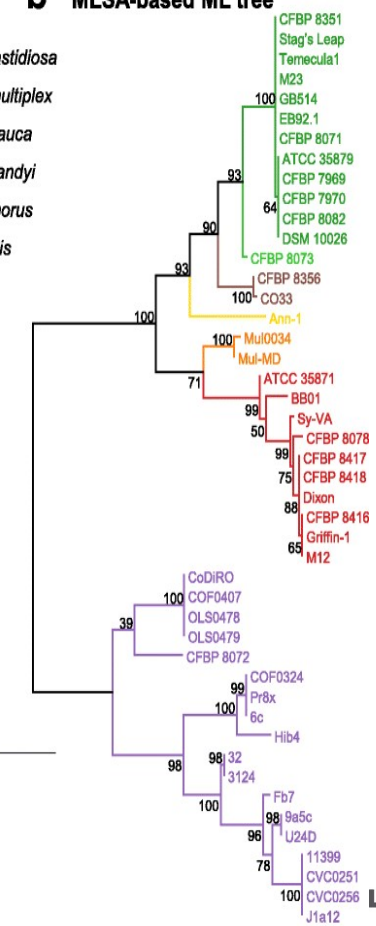
Phylogeny -whole genome sequencing and MLSA

a Whole genome-based dendrogram



b MLSA-based ML tree

- *X.f. subsp. fastidiosa*
- *X.f. subsp. multiplex*
- *X.f. subsp. pauca*
- *X.f. subsp. sandyi*
- *X.f. subsp. morus*
- *X. taiwanensis*



Amplicon Sequencing- MinION

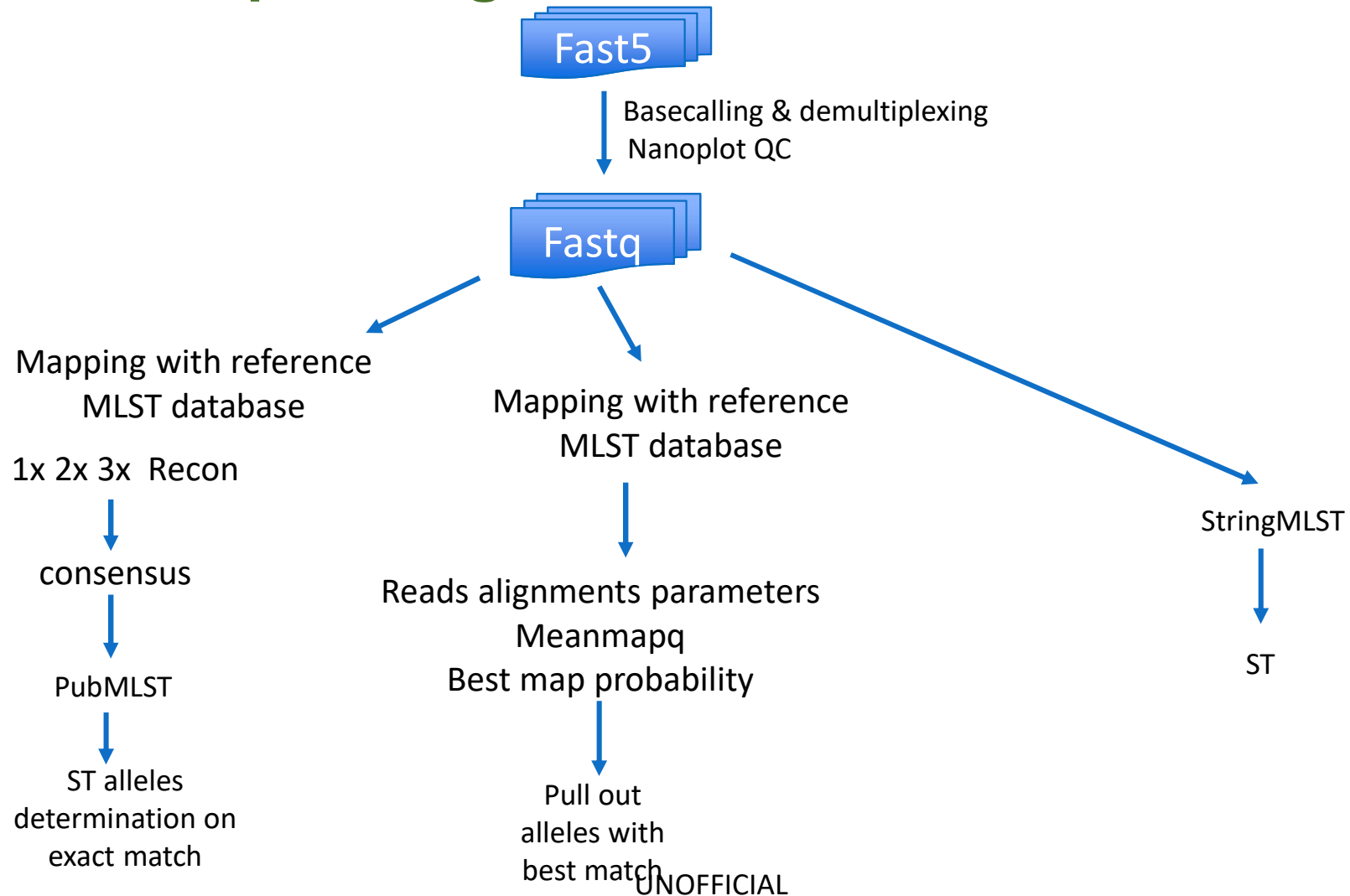


Table 8: ONT-MLST amplicon sequence data outputs for *Xylella fastidiosa* DNA spiked into Olive DNA at different concentrations and results for sequence type (ST) assignment using StringMLST and read mapping using MiniMap2.

Concentration of <i>Xylella</i> DNA (ng/ μ L)	Mean read length	Mean read quality	Number of reads	Median read length	Read length N50	String-MLST	MiniMap Aligned reads	MiniMap ST
Olive +2.4 x 10 ⁻⁰⁶	798	10.5	295,286	557	689	ST 2	110,229	ST2
Olive 2.4 x 10 ⁻⁰⁷	798	10.5	295,286	557	689	UD	60,477	ST2
Olive 2.4 x 10 ⁻⁰⁸	826	10.5	188,908	537	905	UD	3,396	UD
Olive 2.4 x 10 ⁻⁰⁹	805	10.5	239,954	553	790	UD	2,528	UD
Olive 2.4 x 10 ⁻¹⁰	774	10.4	337,964	535	850	UD	2,086	UD
Olive 2.4 x 10 ⁻¹¹	841	10.5	625,954	536	936	UD	2,114	UD
Olive 2.4 x 10 ⁻¹²	787	10.5	372,002	535	793	UD	2,196	UD
water	685	10.4	114,224	585	633	UD	2,297	UD



Diagnostic preparedness for the threat posed by *Xylella* in Australia: In-field detection tools

AgVIC: Rachel Mann, Pragya Kant, Fiona Constable, **DPI NSW:** Toni Chapman, Johanna Wong, **MPI NZ:** Robert Taylor, Luciano Rigano, **QDAF:** Cherie Gambley, Rebecca Roach, **DPIRD:** Monica Kehoe,



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**Horticulture
Innovation**

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Xylella is Australia's number one NPPP

- Xylem limited bacteria with a broad host range
 - *Xylella fastidiosa* (>650 host species)
 - *Xylella taiwanensis* (only reported on *Pyrus pyrifolia*)
- *Xylella spp* are not present in Australia or New Zealand
- In-field diagnostics:
 - Easily deployable assays that can be used in-field for rapid results
 - Can be used as a triage tool on symptomatic plants as part of surveillance



Temecula vineyard (2000) destroyed by Pierce's disease.



Photo: Fiona Constable - Almond leaf scorch



October 2013



March 2016

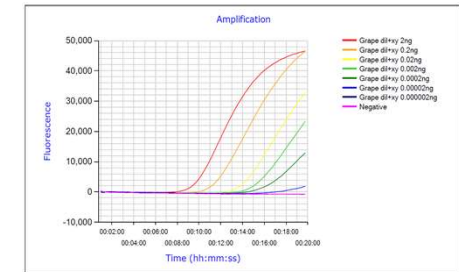
Photo: D. Boscia PLOS Biology 2018

In-field detection of *Xylella*



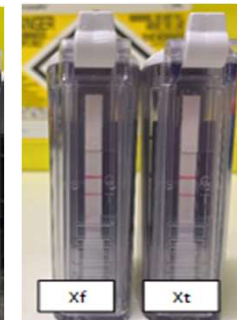
Goal: In-field detection capability ready for application in the field by field officers included in a revised Australian NDP for *Xylella*

- Isothermal in-field assays (LAMP and RPA) identified as the technology most field ready and transferable to field officers
- Six different isothermal assays were assessed in an Australian setting (including assays designed in this project). Assessed on:
 - Specificity
 - Sensitivity
 - Ease of use by operator
- Recommendation for inclusion in the NDP:
 - LAMP of Harper et al (2010) (Real-time)
 - As published
 - Optigene kit
 - Agdia Amplify RP® XRT kit



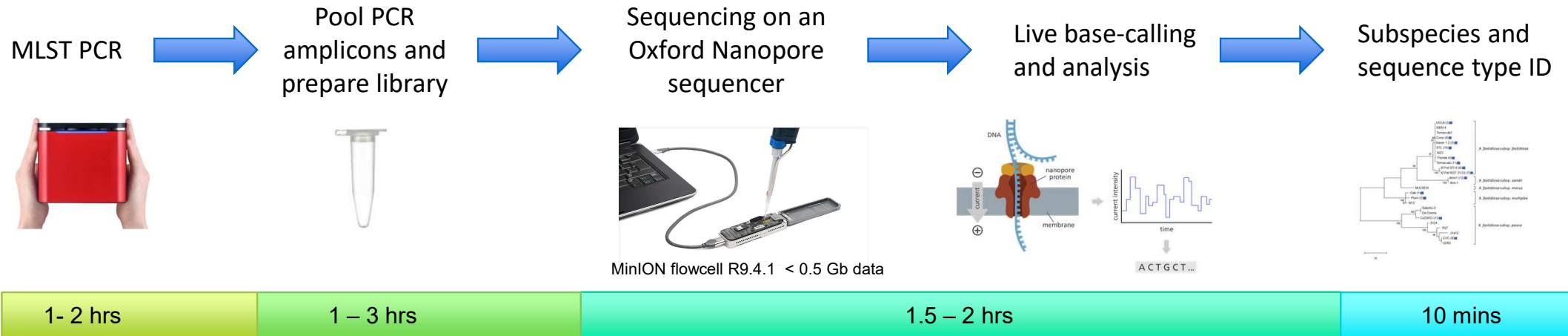
DNA		Harper qPCR	Optigen Xf LAMP kit		Harper LAMP		Agdia	
Conc	Sample Name	Ct Mean	Tp	AD	Tp	AD	RPA Tp	LFD
2.40E-01	XFPD1	21.7	13.9	89.5	7.2	88.7	5:30	Positive
2.40E-02	XFPD2	24.7	16.7	89.4	8.9	88.7	8:00	Positive
2.40E-03	XFPD3	28.1	23.9	88.9	15.4	88.4	13:15	Positive
2.40E-04	XFPD4	31.9	UD	UD	14.9	87.9	UD	UD
2.40E-05	XFPD5	35	UD	UD	22.2	87.9	UD	UD
2.40E-06	XFPD6	37.6	UD	UD	UD	77.5	UD	UD
2.40E-07	XFPD7	UD	UD	UD	UD	77.4	UD	UD

100% specificity against 149 samples
10 -1000 x less sensitive than qPCR



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Detection of *Xylella* – ONT amplicon sequencing



Spiked concentration of Xf DNA		qPCR assays		LAMP assays	Metagenomic seq			ONT-MLST amplicon seq
		Haper et al 2010	Dupas et al 2019 (generic)	Harper et al 2010 (Tp)	2 GB	5 GB	10 GB	
No dilution	240 pg/μl	21.90	26.38	9.9	ST2	ST2	ST2	Not tested
10 ⁻¹	24 pg/μl	24.52	29.46	11.2	XF	XF	ST2	Not tested
10 ⁻²	2.4 pg/μl	27.87	33.17	12.9	UD	UD	XF	ST
10 ⁻³	240 fg/μl	31.17	36.16	14.7	UD	UD	UD	ST
10 ⁻⁴	24 fg/μl	34.21	39.00	20.4	UD	UD	UD	ST
10 ⁻⁵	2.4 fg/μl	37.45	UD	UD	UD	UD	UD	ST
10 ⁻⁶	240 ag/μl	UD	UD	UD	UD	UD	UD	ST
10 ⁻⁷	24 ag/μl	UD	UD	UD	UD	UD	UD	XF
10 ⁻⁸	2.4 ag/μl	UD	UD	UD				UD

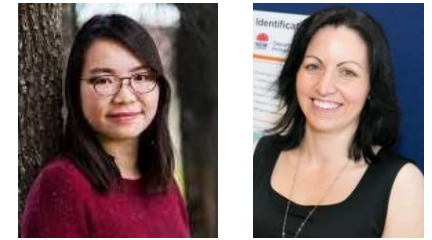
Complete in one day



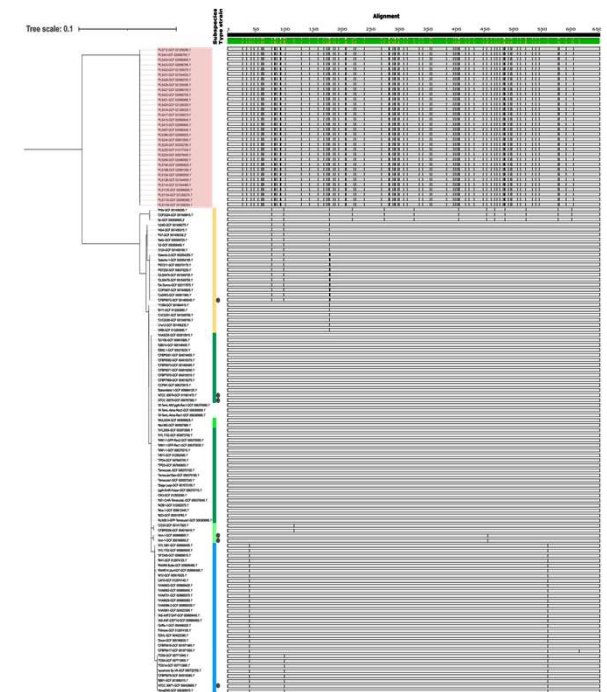
Dr Pragya Kant

Detection of *Xylella* – ONT amplicon sequencing

- A new genome-informed *Xylella* generic endpoint PCR has been designed and broadly validated in an Australian setting
 - 100% specificity across approx. 200 samples
 - PCR sensitivity approx. 100 x lower than qPCR
- The amplicon generated from this PCR can be used to differentiate species of *Xylella* and the subspecies of *X. fastidiosa*
- ONT sequencing of this product in Australia and New Zealand has been used to accurately identify *Xylella* from infected plant tissue to the species and subspecies level
- Sensitivity testing of the ONT amplicon sequencing of this product is underway – likely to be equal to qPCR
- ONT sequencing of the amplicon generated from this PCR is highly adaptable to in-field testing

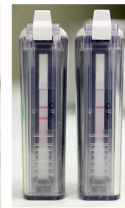


Dr Johanna Wong and Dr Toni Chapman
NSW DPI



Project outcomes

- In-field detection capability ready for application in the field by field officers - including easy to use commercially available kits.
- LAMP and RPA assays broadly evaluated and validated for use in an Australian setting. Both tests provided 100% specificity against 149 samples providing confidence in their use in Australian conditions and supporting their inclusion in the Australian NDP.
- Development of a highly sensitive detection tool for identification of *Xylella in planta* to the species and subspecies level (generic endpoint PCR-ONT) and to the sequence type level (MLST- ONT). Both assays are field-deployable.



Ellen de Vries doing in-field ONT amplicon sequencing for avian influenza (AgVIC)



Brendan Rodoni running LAMP training for biosecurity officers (AgVIC)

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The journey to develop new and updated National Diagnostic Protocols

- Key Learnings from Citrus Canker and
Xylella spp. NDPs

National Diagnostic Protocol for Asiatic Citrus Canker,
Xanthomonas citri subsp. *citri*



The International Diagnostic Protocol for *Xanthomonas Citri* subsp. *Citri* (ISPM-27 DP06) was released August 2014. <https://www.ippc.int/publications/dp-6-2014-xanthomonas-citri-subsp-citri>

PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 9
VERSION NUMBER	V1.2
PROTOCOL STATUS	Endorsed
ISSUE DATE	November 2011
REVIEW DATE	2016
ISSUED BY	SPHDS

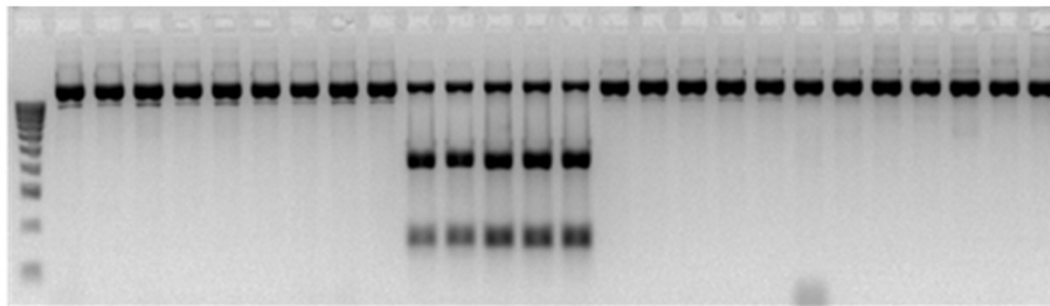


- UNOFFICIAL
False positives generated with both molecular assays
- We developed a multiplex assay and LAMP specific to *X. citri* subsp *citri*
- The multiplex had two internal controls 16S and COX
- Conducted specificity assays on DNA, and plant material that was preserved in ethanol
- Conducted limit of detection assays
- Protocol was submitted for verification
- Three days later citrus canker was detected in the NT
- Verification of the protocol was conducted on suspect samples during the incursion

What happened next....

- NT and NSW conducted assays using both the existing NDP and the new draft NDP
- One leaf sample had both fungal growth and citrus canker, the fungus overwhelmed the amplification of the COX, which resulted in non-amplification of the *X. citri* subsp *citri* and the 16S band, only COX amplified
- Because of this, the result was considered a false negative, the COX was removed from the multiplex and revalidated with the same DNA samples

X. citri subsp. *malvacearum* *X. citri* subsp. *citri* *X. citri* subsp. *mangiferaeindicae*

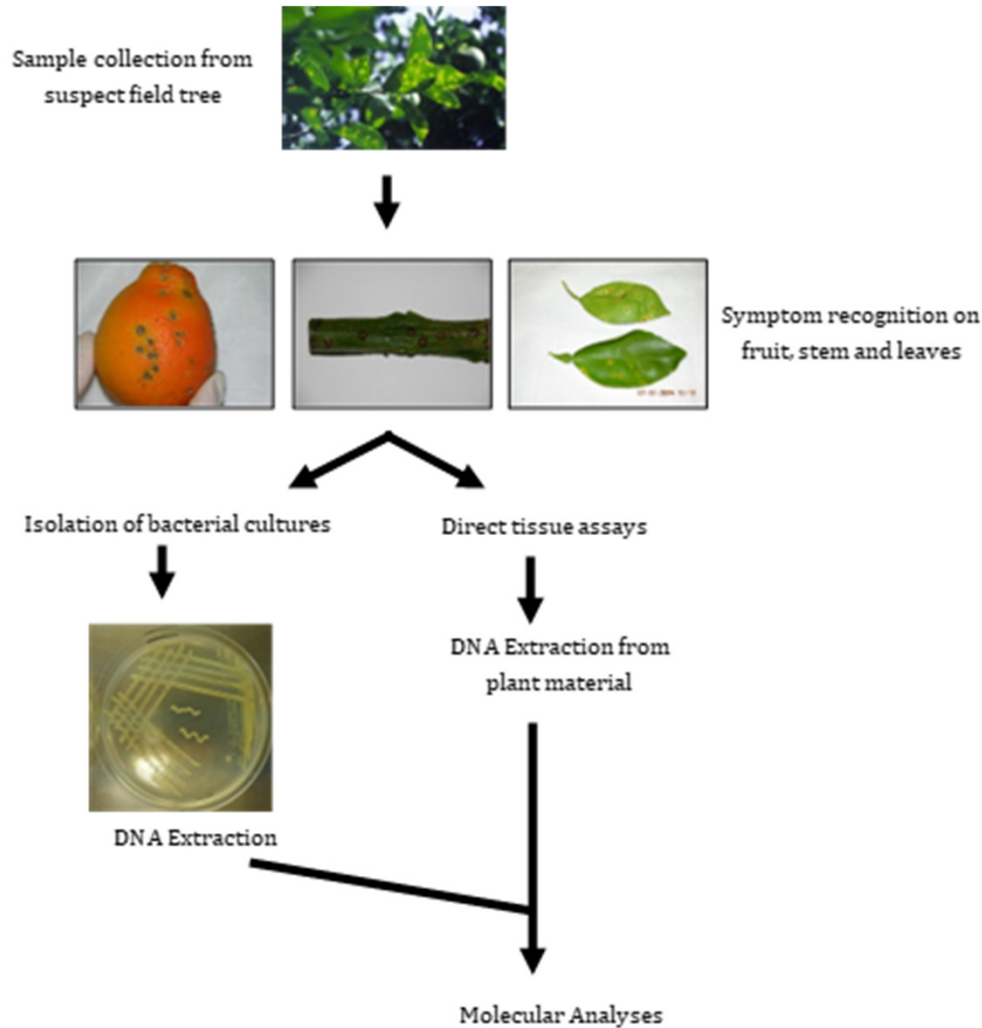


16S fragment 1300 bp

AAA Domain Protein (R22) 400 bp

Intergeneric Region (R8) 200 bp

Learnings from this experience



- A descriptive diagnostic flow chart needs to be provided, this is now the new flow chart, however it will now so be similar to the *Xylella* spp.
- Inclusion of a description of what the results mean with internal controls and what to do when there are mixed results from the two assays
- Addition molecular assays for confirmation, preferable ones that can be sequenced

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Xylella spp.

The journey to develop a new National
Diagnostic Protocol

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Xylella spp. – Australia's No1 National Priority Plant Pest

1 genus: *Xylella*

2 species: *X. taiwanensis* (Responsible for Pear leaf scorch)

X. fastidiosa (Responsible for bacterial leaf scorch in many hosts)

6 subspecies: subsp. *fastidiosa*

subsp. *multiplex*

subsp. *pauca*

subsp. *sandyi* * (*X. fastidiosa sensu largo*)

subsp. *morus* * (*X. fastidiosa sensu largo*)

subsp. *tashke* * (*X. fastidiosa sensu largo*)

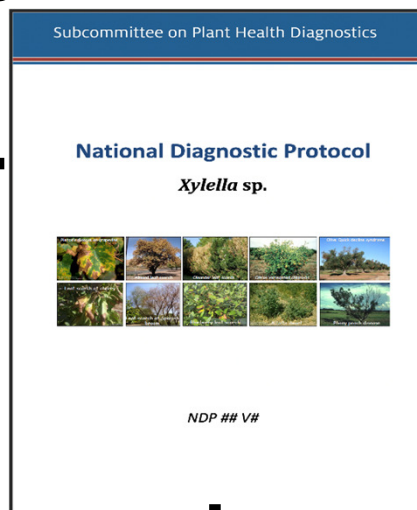
* Genomic data doesn't support these subspecies.

* No original strains available for confirmation

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Incorporating of the citrus canker NDP learnings into the *Xylella* spp. NDP journey

Processing of samples & isolation of bacterial cultures



Section 9 – surveillance samples

Generic *Xylella* Endpoint PCR assay, with sequence confirmation

Molecular assays

Genome sequencing

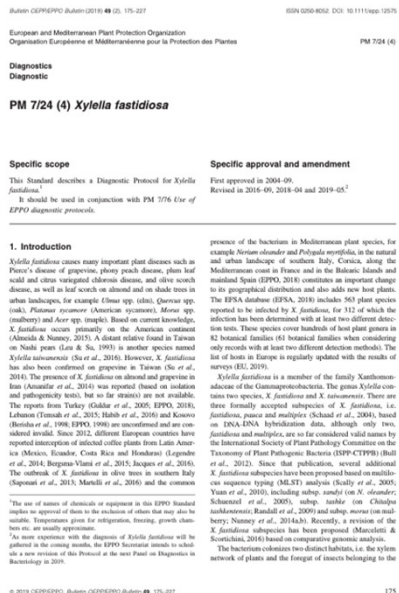
Xylella qPCR assay

Subspecies identification multiplex qPCR

Multi-locus Sequence Typing (MLST)

Differentiation between *X. fastidiosa* and *X. taiwanensis*

Where to start



- Testing of protocols in the EPPO and IPPC documents
- Testing of recent protocols in the literature
- Development of a generic *Xylella* assay

Assays Undergoing Testing

- Four Endpoint PCR's: Minsavage 1994
Ito & Chiaki 2021
Two generic *Xylella* spp. 2021 (Generic 1 & Generic 2)
- Nine qPCR's: Harper 2010
Harper 2010 LAMP
Agiletti 2019
Ouyang 2013
Li 2013
Francis 2006
Ito & Suzuki 2017
qPCR uniplex 2021 (Targets *Xylella* spp.)
qPCR multiplex 2021 (Targets *Xylella* spp., Xf & Xt)
- Two subspecies qPCR's: Dupas 2019
Hodgetts 2020

Testing Panel

- 31 *Xylella*
- 16 *Xanthomonas*
- 9 *Stenotrophomonas*
- 18 *Xylella* positive plant samples
- 1 healthy plant sample
- 6 *Xylella* positive insect samples

Sensitivity Panel

- *Xylella fastidiosa* subsp *fastidiosa*
- *Xylella fastidiosa* subsp *multiplex*
- *Xylella fastidiosa* subsp *pauca*
- *Xylella taiwanensis*

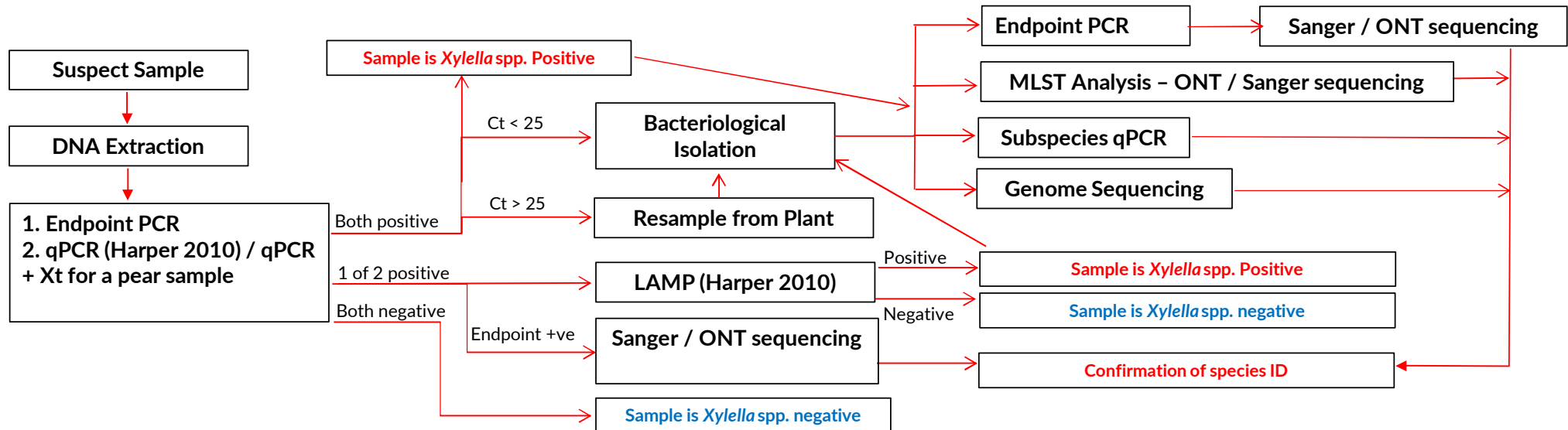
UNOFFICIAL	Name	ID Code
	<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 8731
	<i>Xylella fastidiosa</i> subsp <i>multiplex</i>	ICMP 8739
	<i>Xylella fastidiosa</i> subsp <i>multiplex</i>	ICMP 8740
	<i>Xylella fastidiosa</i>	ICMP 8742
	<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 8745
	<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	ICMP 15197
	<i>Xylella fastidiosa</i> subsp. <i>piercei</i>	NCPPB4432 / ICMP 15197
	<i>Xylella fastidiosa</i> subsp. <i>multiplex</i>	NCPPB4604 / BB164
	<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8495 / PD7202
	<i>Xylella fastidiosa</i> subsp <i>fastidiosa</i>	CFBP8071
	<i>Xylella fastidiosa</i> subsp. <i>sandyi</i>	CFBP8524 / C033
	<i>Xylella fastidiosa</i>	CFBP8173
	<i>Xylella fastidiosa</i>	CFBP7969
	<i>Xylella fastidiosa</i>	CFBP7970 / ICMP 15197
	<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8477 / Salento-1
	<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8072
	<i>Xylella fastidiosa</i>	CFBP8073
	<i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i>	CFBP8082 / ICMP 8745
	<i>Xylella fastidiosa</i> subsp. <i>pauca</i>	CFBP8498 / PD7211
	<i>Xylella fastidiosa</i>	NCPPB4605 / Temecula1
	<i>Xylella fastidiosa</i> subsp. <i>sandyi</i>	CFBP8077 / Ann-1
	<i>Xylella taiwanensis</i>	NCPPB4612
	<i>Stenotrophomonas</i> sp.	DAR 65801
	<i>Stenotrophomonas</i> sp.	DAR 72045
	<i>Stenotrophomonas</i> sp.	DAR 75512
	<i>Stenotrophomonas</i> sp.	DAR 76132
	<i>Stenotrophomonas</i> sp.	DAR 77232
	<i>Stenotrophomonas</i> sp.	DAR 77233
	<i>Stenotrophomonas</i> sp.	DAR 77234
	<i>Stenotrophomonas</i> sp.	DAR 77236
	<i>Stenotrophomonas</i> sp.	DAR 77237
	<i>X. fuscans</i> subsp <i>aurantifolii</i>	1622 B Strain
	<i>X. alfalfae</i> subsp <i>citrumelo</i>	P03- 83
	<i>X. vasicola</i>	DAR41379
	<i>X. vesicatoria</i>	DAR73877
	<i>X. translucens</i> pv <i>translucens</i>	DAR35705
	<i>X. hortorum</i>	DAR49849
	<i>X. arboricola</i>	DAR33337
	<i>X. campestris</i> pv <i>phaseoli</i>	DAR82645
	<i>Xanthomonas</i> sp.	DAR82580
	<i>X. oryzae</i>	DAR82611
	<i>X. campestris</i> pv <i>vitians</i>	DAR30526
	<i>X. campestris</i>	VPRI41552
	<i>X. campestris</i> pv <i>pruni</i>	DAR82627
	<i>Xanthomonas</i> sp.	USA 3- 8A.1
UNOFFICIAL	<i>X. cucurbitae</i>	DAR82711
	<i>Xanthomonas</i> sp.	DAR72015

Additional validation

- Plant samples from various hosts across NSW, Vic, Qld and WA labs have been validated
- DNA extraction testing will occur next for incorporation into the NDP
- Proficiency assays have been conducted at all group labs
- NDP has been reviewed by all group members
- NDP will now be read by two additional people

Improvements in the *Xylella* spp. NDP

- Inclusion of an improved diagnostic flow chart



- Inclusion of written text explaining what results should be and what to do next, especially if there are questionable results
- Inclusion of molecular assays for additional confirmation
- Inclusion of assays for subspecies identification, MLST and ONT-sequencing

Acknowledgements

- Horticulture Innovation for project funding – MT17006
- PBCRC for funding the citrus canker work
- Adrian Dinsdale and DAWE team for additional testing material

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Nerida Donovan
Grant Chambers

Victoria

Rachel Mann – Project Lead
Pragya Kant
Elisse Nogarotto
Fiona Constable

New Zealand - MPI

Robert Taylor
Luciano Rigano

Queensland

Cherie Gambley
Rebecca Roach

WA

Monica Kehoe
Domine Wright



Targeted sequencing approaches for *Xylella* identification and surveillance

Dr Pragya Kant
Research Scientist

High Throughput Sequencing (HTS) for pathogen detection (metagenomic)

DNA of *Xylella fastidiosa* subsp. *fastidiosa* (Xff) was serially diluted into olive DNA.

LAMP, qPCR and metagenomic sequencing was run on Xff standards.

- Results showed qPCR was 1000-10,000 times more sensitive than metagenomic studies (<10GB)
- Identification of Xff in GB

Limitation – Host DNA is predominately sequenced- waste of resources, specifically for low-titre, unculturable and fastidious bacteria

Way-out is Enrichment- Probes and Amplicon seq

Concentration of <i>Xylella fastidiosa</i> DNA (ng)	24.5	11.2	XF	XF	ST2	2089	4686	9542	133	488	1233	690	1570	3106	1.84	7.23	20.59
2.4 x 10 ⁻⁰²	24.5	11.2	XF	XF	ST2	2089	4686	9542	133	488	1233	690	1570	3106	1.84	7.23	20.59
2.4 x 10 ⁻⁰³	27.9	12.9	UD	UD	XF	190	501	1155	3	11	45	523	439	575	0.05	0.142	0.619
2.4 x 10 ⁻⁰⁴	31.2	14.7	UD	UD	UD	13	57	96	1	1	1	317	281	333	0.012	0.011	0.013
2.4 x 10 ⁻⁰⁵	34.2	20.4	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁶	37.5	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁷	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
2.4 x 10 ⁻⁰⁸	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

AVR has developed two targeted strategies using HTS

- Hybridization Probe – applicable at the PEQ to target National Plant Priority Pest list

Xylella spp

Erwinia amylovora

Phytoplasmas

Xanthomonas citri subsp. citri

Liberibacter

- Amplicon HTS – applicable at the time of surveillance and an outbreak of *Xylella fastidiosa* detection and sequence typing

Both methods have been applied for *Xylella* spp. detection with increased or equivalent sensitivity than a qPCR

High Throughput Sequencing (HTS) for bacterial pathogen detection

- Aim was to use HTS to detect all phytopathogenic bacteria on the National Priority Plant Pests (NPPP) list in one test (consisting of 12 species/subspecies across 5 genera)
- Many of these are low titre, fastidious, non-culturable
- Multiple PCRs/Sanger sequencing for detection and identification
- What do we want from using HTS:-
 - prof of concept
 - Detection of all pathogens in a sample
 - Retain or improve sensitivity of current tests
 - Make cost effective



Erwinia amylovora



Xanthomonas citri
subsp. *citri*



Xylella
fastidiosa



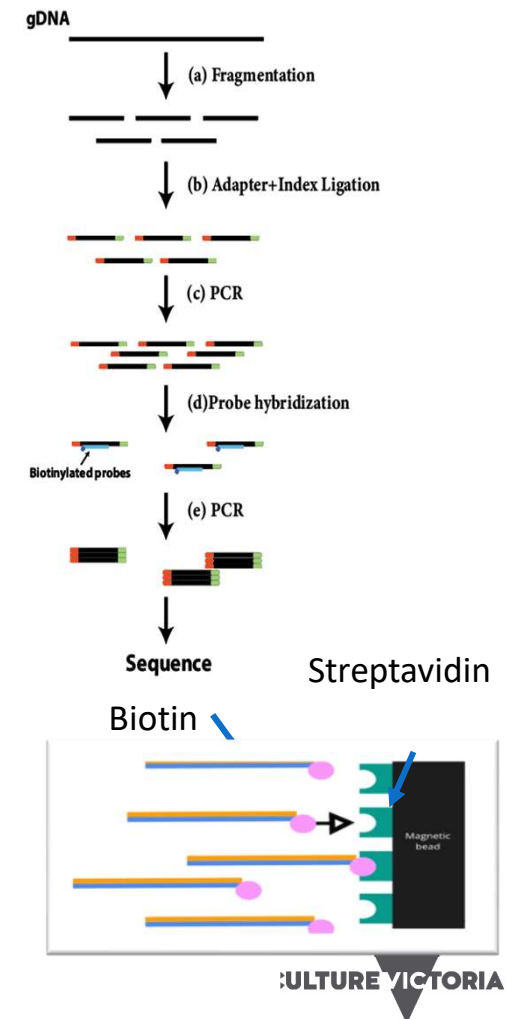
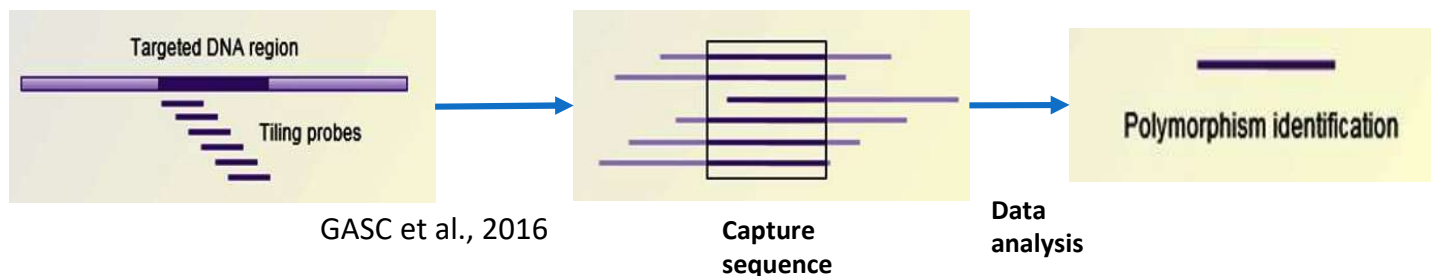
Candidatus
phytoplasma
16Sr1



Ca. *Liberibacter*
asiaticus

High Throughput Sequencing (HTS) for pathogen detection

- Literature and databases were searched and compared for putative target regions
 - MLSA/MLST genes
 - Diagnostic target regions
 - Genes associated with biosynthetic pathways
 - 3071 probes specifically for *Xylella*
 - 15000 probes for 5 genera



Increased sensitivity to the target regions in metagenomic samples

Sample Name	qPCR	<i>Xylella fastidiosa</i> subsp.	Probed	On-target reads	On-target percentage (%)
<i>Xylella</i>					
Vitis B1-2	23	<i>fastidiosa</i>	Yes	1292502	47.31
			No	215	0.0041
Vitis B2-3	24	<i>fastidiosa</i>	Yes	5767959	44.72
			No	211	0.0023
Vitis B2-7	30	<i>fastidiosa</i>	Yes	436552	36.05
			No	124	0.0013
Olea B3-1	23.7	<i>sandyi</i>	Yes	19887876	64.44
			No	65	0.0013
Olea B4-2	25.3	<i>sandyi</i>	Yes	12970131	60.93
			No	29	0.0008
NGS XF Vitis-1	24.6	<i>fastidiosa</i>	Yes	17043697	59.35
			No	76	0.0020
NGS XF Vitis-2	26.7	<i>fastidiosa</i>	Yes	22215079	59.73
			No	73	0.0014
Spiked ICMP 8739	27.04	<i>multiplex</i>	Yes	2273256	57.74
			No	129	0.0016
Spiked CFBP 8072	30.57	<i>pauca</i>	Yes	248600	26.11
			No	18	0.0002
Spiked NCPPB 4612	-	<i>Xylella taiwanensis</i>	Yes	263290	41.30
			No	15	0.0002

Sensitivity of hybridization probes - XFF

Concentration of Xff DNA (ng/μL)	LAMP (amp time)	Non-probed		Probed		Fold enrichment	qPCR (Ct)
		Detected	MLST	Detected	MLST		
0.24	9.9	D	ST	D	ST	5,608	21.09
2.4×10^{-02}	11.2	D	XF ¹	D	ST	84,370.4	24.52
2.4×10^{-03}	12.9	-	-	D	ST	122,206.3	27.87
2.4×10^{-04}	14.7	-	-	D	ST	23,597.2	31.17
2.4×10^{-05}	20.4	-	-	D	ST	45,796.9	34.21
2.4×10^{-06}	UD	-	-	D	XF ¹	1,963.5	37.45
2.4×10^{-07}	UD	-	-	-	-	*	UD
2.4×10^{-08}	UD	-	-	-	-	0.6	UD

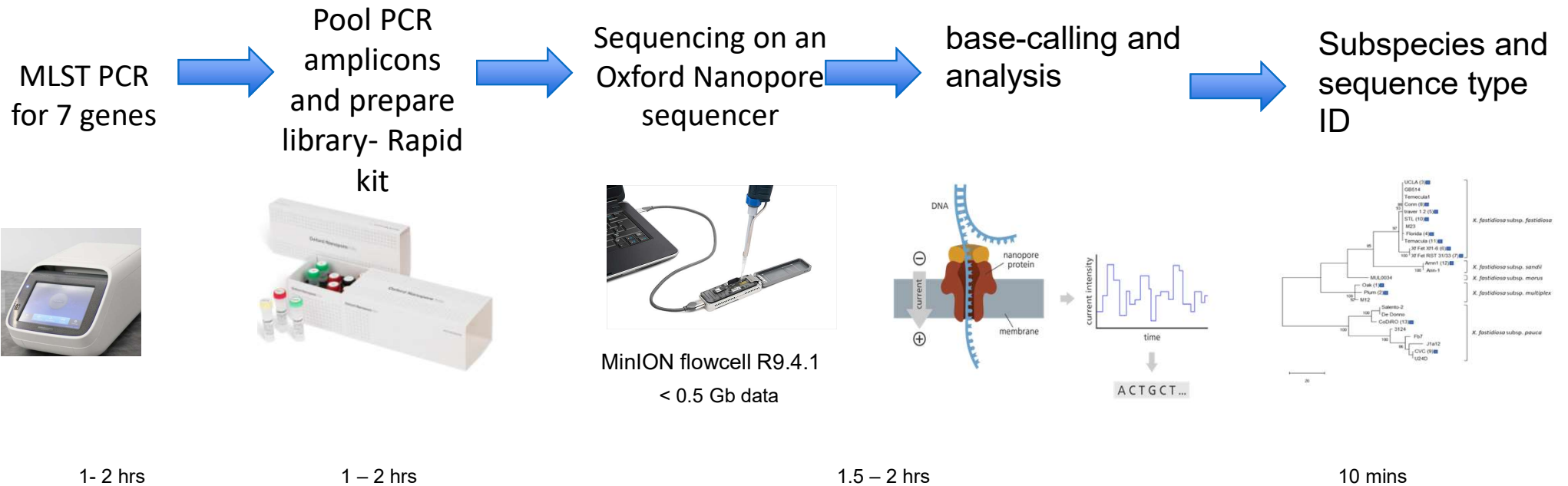
- Same level as qPCR
- Identification of ST at Ct 34.2

Multi-locus sequence typing- To characterise *Xylella fastidiosa*

- 7 genes for *X. fastidiosa* *cys*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC* (Sclly et al 2005)
- Diff
- Exa **In case of an incursion- it is valuable to** fast.org is trees
- in S **know Sequence Type ASAP**
- *Xf pauca* (main hosts: Citrus and Coffea spp.)
- Sequence typing found it was different ST 53
- STs are essential as they are associated with host specificity and introduction

Detection of *Xylella* – MLST amplicon sequencing

Similar work flow to Fiano et al. 2019



Completed in a day

Increased sensitivity of detection with enrichment

Spiked concentration of Xf DNA in Olive		qPCR	LAMP	Metagenomic seq			Enrichment	
		Haper et al 2010	Harper et al 2010 (Tp)	2 GB	5 GB	10 GB	MLST-amplicon seq	Hyb. Probes
No dilution	240 pg/ μ l	21.90	9.9	ST2	ST2	ST2	ST	ST
10 ⁻¹	24 pg/ μ l	24.52	11.2	XF	XF	ST2	ST	ST
10 ⁻²	2.4 pg/ μ l	27.87	12.9	UD	UD	XF	ST	ST
10 ⁻³	240 fg/ μ l	31.17	14.7	UD	UD	UD	ST	ST
10 ⁻⁴	24 fg/ μ l	34.21	20.4	UD	UD	UD	ST	ST
10 ⁻⁵	2.4 fg/ μ l	37.45	UD	UD	UD	UD	ST	XF
10 ⁻⁶	240 ag/ μ l	UD	UD	UD	UD	UD	XF	UD
10 ⁻⁷	24 ag/ μ l	UD	UD	UD	UD	UD	UD	UD
10 ⁻⁸	2.4 ag/ μ l	UD	UD				UD	UD

- ✓ MLST-seq 10 fold more sensitive than qPCR
- ✓ Hybridization probes had same sensitivity as qPCR

Conclusions

Hybridization Probes

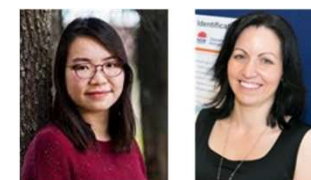
- Proof of concept was highly successful
- Proven to increase detection limits of HTS protocols to PCR and qPCR levels
- Probes for MLST genes can provide extra vital information; ST, haplotype, origin etc.
- Could be used to produce priority pathogen panels for specific commodities
- Will reduce testing time in PEQ
- Cost effective

MLST amplicon sequencing

- Amplicon sequencing through Nanopore sequencing is the fastest method to know species, subspecies and sequence types.
- This approach is more sensitive to qPCRs.
- MLST amplicon sequencing have been added to the updated NDP.
- Proficiency testing was done by all key node laboratory of Australia and New Zealand and has capacity and capability for fast detection of ST

HTS combined with enrichment is a powerful tool for rapid and robust detection of low-tire and fastidious pathogens.

Natasha Brohier
 Rachel Mann
 Brendan Rodoni
 Fiona Constable
 Elisse Nogarotto
 Bianca Rodrigues Jardim
 Tongda Li
 Rebecca Roach
 Monika Kehoe
 Luciano Rigano
 Robert Taylor



Drs Johanna Wong and
Toni Chapman, NSW DPI



Ministry for Primary Industries
Manatū Ahu Matua



Australian Government
Department of Agriculture,
Water and the Environment



Department of
Primary Industries and
Regional Development



MT18005 project team from QUT, NZ MPI
 and DAWE PEQ
 MT17006 project team from VIC, NSW, Qld,
 WA and NZ

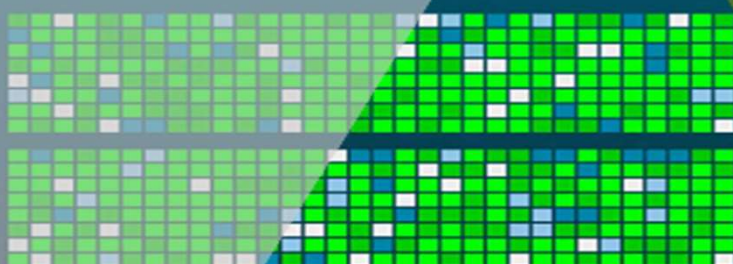
OFFICIAL

Multi-locus target sequencing (MLST) workshop

Dr Pragya Kant
Research scientist
AgriBio, AVR

Channel states panel

Run state: sequencing



**Hort
Innovation**

AGRICULTURE VICTORIA

Contents

Theory

- Xylella Pathogen
- Sequence typing - MLST- PubMLST
- Steps for MLST amplicon seq
- Mk1C and MinION device
- MinKNOW software

Hands-on

- Data analysis
- Flow cell handling- Priming, Flushing, Loading



 YouTube
Introducing MinION Mk1C - YouTube

Background

- *Xylella* spp. bacteria that causes disease by blocking xylem within the host
 - Transmitted by Hemiptera species
 - Sharpshooter, leafhoppers, spittlebugs
 - Inhabits the foregut of insect vectors
 - Broad host range. Infects well over 600 plant species
 - Symptoms are difficult to detect, as 'scorch' symptoms are common
 - Low-titre pathogen, may not show symptoms
 - No control options
-
- ✓ *Xylella* not present in Australia and New Zealand
 - ✓ *Xylella* is #1 Australia's National Priority Plant Pest



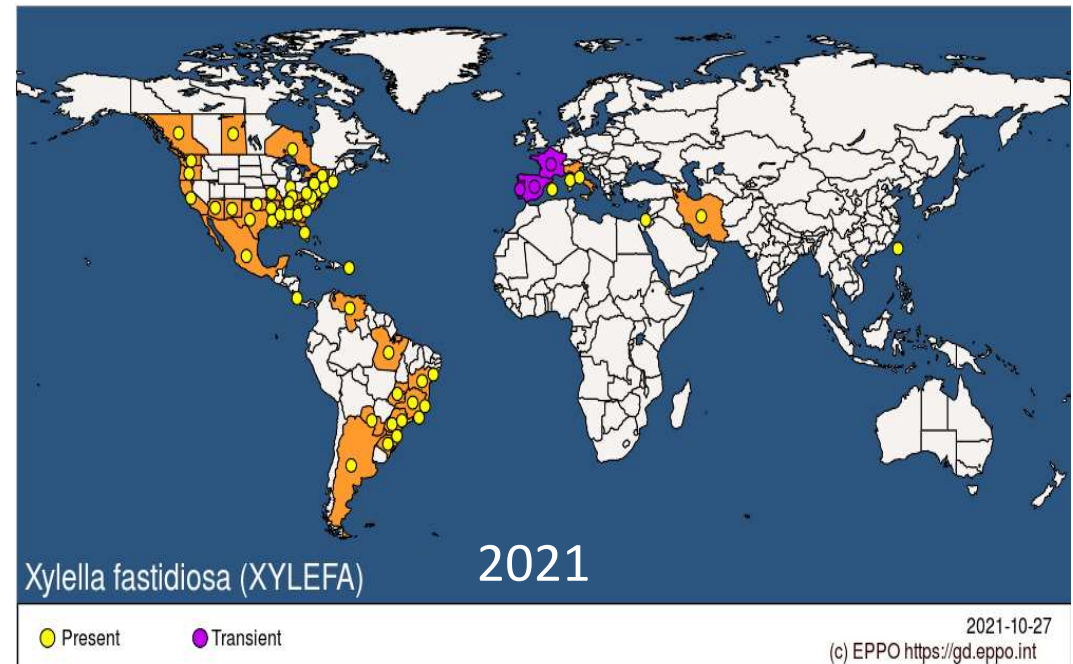
SEM of Xf filled xylem of a coffee petiole © Jose Lima



Expanding Host Range and Distribution

	2016	2018	2020	2021
Plant species reported infected	359	563	638	664
Genera	204	264	289	299
Botanical families	75	82	87	88

EFSA Journal 2016;14(2):4378
 EFSA Journal 2018;16(9):5408
 EFSA Journal 2021;19(6):6674



- Currently in the Americas, Spain, Italy, France, Iran, Israel and Taiwan
- Eradication? Possible/not possible if vector is persistent

Xylella Taxonomy

Genera *Xylella*

Species

Xylella fastidiosa (>650 host species)

Xylella taiwanensis (only reported on *Pyrus pyrifolia* in Taiwan)

Six different subspecies of *X. fastidiosa*

- *X. fastidiosa* subsp. *fastidiosa*
 - *X. fastidiosa* subsp. *multiplex*
 - *X. fastidiosa* subsp. *pauca*
 - *X. fastidiosa* subsp. *sandyi*
 - *X. fastidiosa* subsp. *tashke*
 - *X. fastidiosa* subsp. *morus*
- sandyi* & *morus* now considered part of *fastidiosa*,
tashke is not isolated since identified

X. fastidiosa is further classified into Sequence types (ST) based on Multi Locus Sequence Typing

Sequence Typing is important to understand host range

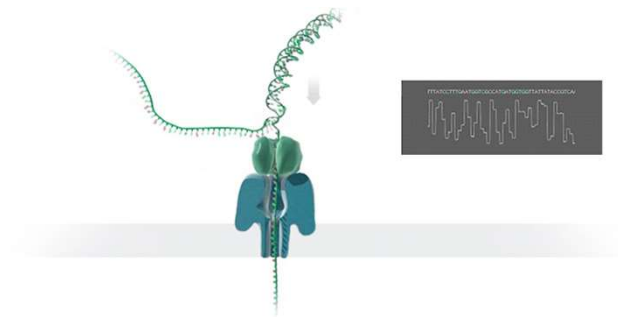
Importance of Sequence Typing

- Sequence types (STs): Multi-locus sequence typing- characterise *X. fastidiosa*
- *X. fastidiosa* MLST by Scally et al 2005, Yuan et al 2010
- 7 housekeeping genes *cys*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC*
- Different alleles for each gene- 156 alleles, 90 known STs (www.pubmlst.org)
- STs are associated with host specificity and introduction
- *X. fastidiosa subspecies pauca*
 - (main hosts: Citrus and Coffea spp.)
 - Strain infected Olives in Italy, identified as Xfp ST53

Advantages of Nanopore sequencing

- High-throughput sequencing platform
- Fast and quick access to data, real-time data output
- Smallest, portable instruments which has lowest instrument and consumables costs.

Long/short DNA molecules → read length



Electric signals are recorded and later converted into bases

Longest confirm reads >100kb but also works for amplicons

Which one to choose!

Sequencing instruments



Cheap \$90. Great for fungal and bacterial work.
Data-500Mb
Single use
Run time 16hr



Good option. Great for fungal and bacterial work.
Data-20Gb
Multiple use
Multiplexing
Run time 72hr



Everything you need in one device for MinION, does Line base calling and data acquisition



5 minion, 100 Gb data real-time
Run time 72hr



24 to 48 flowcells. Lowest price per Gb of data
150Gb data per flowcell
Run time 64hr



On horizon-smallest seq device to use with smart phone in any location

MinKNOW program is required to run all these devices

Nanopore sequencing- resources

<https://nanoporetech.com/>

<https://nanoporetech.com/community>

<https://nanoporetech.com/resource-centre/knowledge>

<https://nanoporetech.com/nanopore-sequencing-data-analysis>

- Various kits for library preparation
- Barcodes for multiple samples
- Stop, wash and reuse a flow cell
- Live base-calling
 - Real-time alignment- cloud based workflows
 - Adaptive sampling- sequence what you want
 - Barcode usage control- sequence samples at variable rates

One limitation – Nanopore reads can contain 5 – 15% errors, new chemistry claims 2% error rates

Xylella fastidiosa Sequence Types

<https://pubmlst.org/organisms/xylella-fastidiosa>

PubMLST

Public databases for molecular typing
and microbial genome diversity

MY ACCOUNT

HOME

ORGANISMS

SPECIES ID

ABOUT US

UPDATES

Home > Organisms > *Xylella fastidiosa* > *Xylella fastidiosa* typing > Download alleles



Download allele sequences

Select loci by scheme | Alphabetical list | All loci by scheme

MLST

Locus	Download	Type	Alleles	Length (setting)	Min length	Max length	Full name/product	Curator(s)	Last updated
leuA		DNA	16	Fixed: 708 bp	708	708		L. Nunney, S. Russell, R. Stouthamer	2022-05-04
petC		DNA	15	Fixed: 533 bp	533	533		L. Nunney, S. Russell, R. Stouthamer	2022-02-18
malF		DNA	20	Fixed: 730 bp	730	730		L. Nunney, S. Russell, R. Stouthamer	2022-02-18
cysG		DNA	36	Fixed: 600 bp	600	606		L. Nunney, S. Russell, R. Stouthamer	2022-02-18
holC		DNA	27	Fixed: 379 bp	379	379		L. Nunney, S. Russell, R. Stouthamer	2022-02-18
nuoL		DNA	24	Variable: (527 min; 557 max)	527	557		L. Nunney, S. Russell, R. Stouthamer	2022-02-18
gltT		DNA	18	Fixed: 654 bp	654	654		L. Nunney, S. Russell, R. Stouthamer	2022-02-18



Contact

Get in touch with us if you have any comments or suggestions concerning the website and the databases.

Cite us

Please cite [Jolley et al. 2018 Wellcome Open Res 3:124](#) if you use data or analysis from PubMLST in your publications.

Follow



Supported by



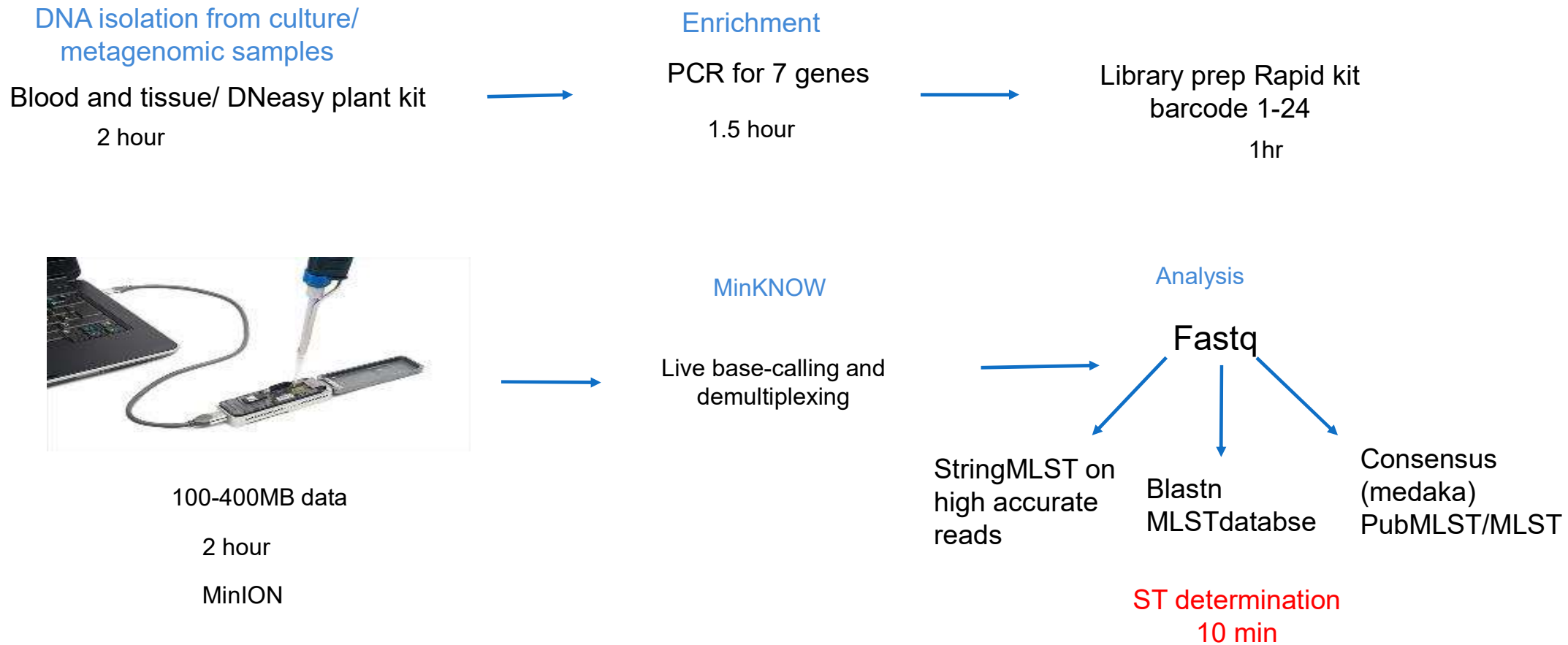
DNA extraction

- Infected plant tissue- plant DNA extracted using the DNeasy Plant Mini Kit (Qiagen)
- Sampling as per NDP recommendations, tissue are collected from Vascular and leaf portions



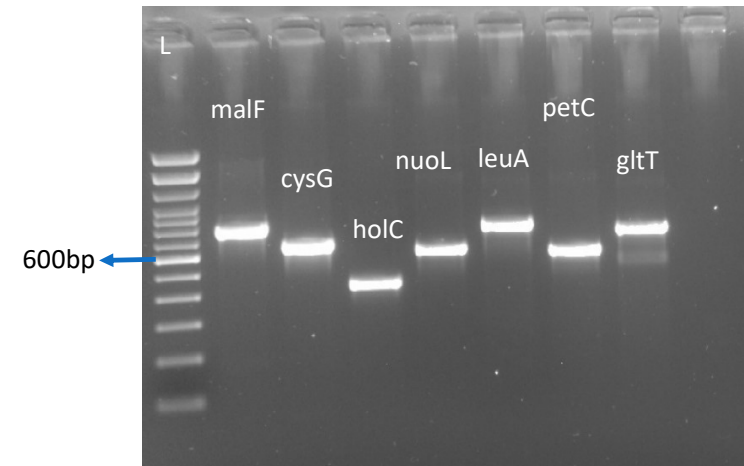
CHS lab, AgriBio, dated 2May 2023

MinION workflow to determine ST



PCR of 7 housekeeping genes-amplicon generation

MLST gene	Primer Name	Sequence (5'-3')	Product size
malF	malF-F	TTGCT GGTCC TGC GG TGTTG	730
	malF-R	GACAG CAGAA GCACG TCCCA GAT	
cysG	cysG-F	GCCGA AGCAG TGCTG GAAG	600
	cysG-R	GCCAT TTTCG ATCAG TGCAA AAG	
holC	holC-F	ATGGC ACGCG CCGAC TTCT	379
	holC-R	ATGTC GTGTT TGTTT ATGTG CAGG	
nuoL	nuoL-F	TAGCG ACTTA CGGTT ACTGG GC	557
	nuoL-R	ACCAC CGATC CACAA CGCAT	
leuA	leuA-F	GGTGC ACGCC AAATC GAATG	708
	leuA-R	GTATC GTTGT GGC GT AACT G	
petC	petC-F	GCTGC CATTC GTTGA AGTAC CT	533
	petC-R	GCACG TCCTC CCAAT AAGCC T	
gltT	gltT-F	TCATG ATCCA AATCA CTCGC TT	654
	gltT-R	ACTGG ACGCT GCCTC GTAAA CC	



Must run a gel to confirm all 7 genes are amplified

95 °C for 2 min, (95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 min) x 35*, 72 °C for 5 min

Library preparation

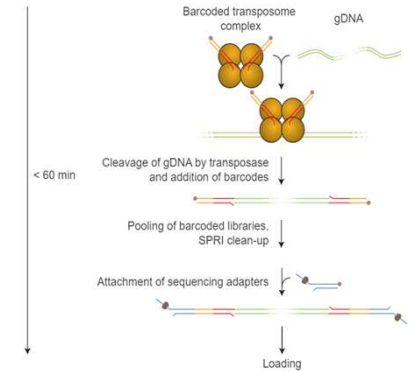
1. Pool all the amplicons in equal concentrations.
2. 400 ng DNA each sample
3. Follow the protocol
 - 7.5 ul DNA
 - 2.5 Barcode
 - Incubate
 - Mix all the barcoded samples
 - Wash and concentrate using magnetic beads
 - Elute
 - Add RAP adapter
 - Library ready
 - Prime the flow cells
 - Load library



1. Quantify each amplicon first time, then you know concentration per ul and take equal volume for pooling.
2. Always quantify for dsDNA.
3. Always start with more DNA as washing steps will lose it
4. Quantify at each step first time so you know magnet beads volumes you are taking are correct
5. Careful never overload library above recommended DNA amounts



Loading the flow cell is tricky, introduction of any air bubble will kill the pores

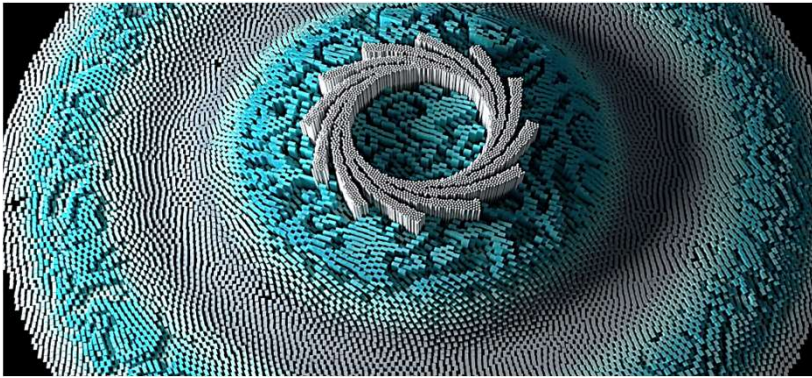


Rapid Barcoding Kits

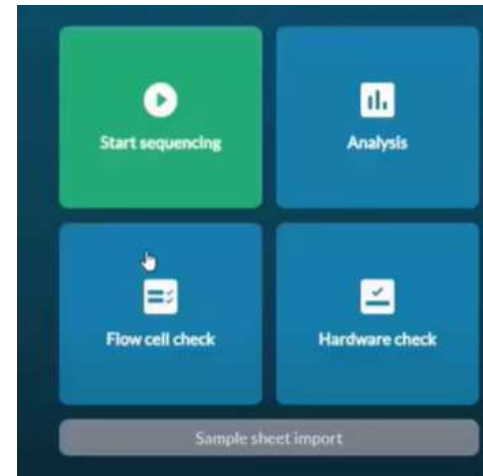
<https://www.youtube.com/watch?v=IknVaEnuDz0>



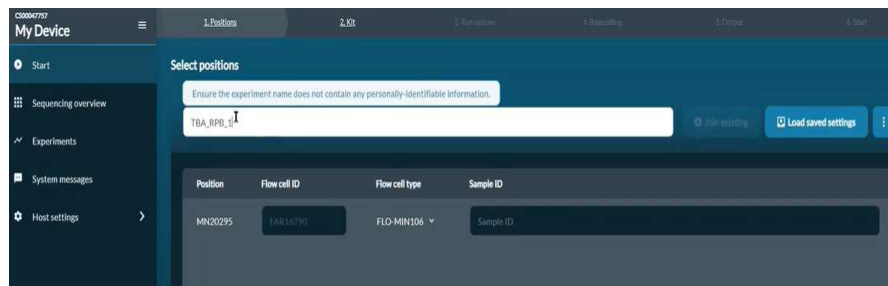
MinKNOW Software



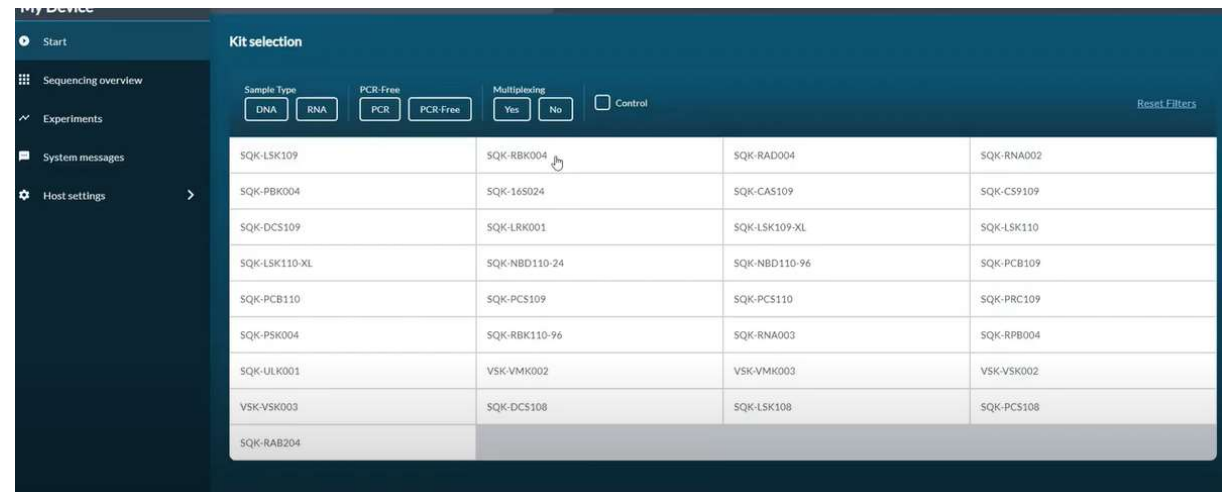
<https://www.youtube.com/watch?v=oCOixfewXo4>



Flow cell check- before loading library < 600 – Replacements

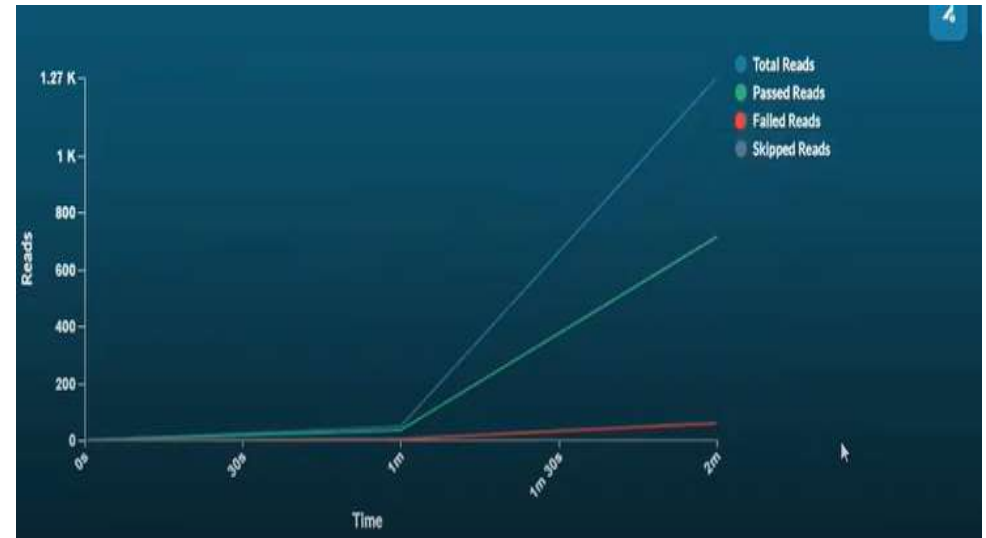


Start seq- fill Experiments, Sample names



OFFICIAL Kits selection – Demultiplexing, option for live base call

MinKNOW Software- a pdf report



Data Analysis

If Fast base calling is done- (Live) recall with super accuracy model

[https://community.nanoporetech.com/downloads#gns\[searchValue\]=guppy](https://community.nanoporetech.com/downloads#gns[searchValue]=guppy)

```
/group/pathogens/Bioinfo_Software/ont-guppy/bin/guppy_basecaller --version
/group/pathogens/Bioinfo_Software/ont-guppy/bin/guppy_basecaller --device cuda:all:100% --num_callers 8 --
config /group/pathogens/Bioinfo_Software/ont-guppy/data/dna_r9.4.1_450bps_sup.cfg --min_qscore 7 --recursive -
-input_path FULL_PATH/fast5/ --save_path FULL_PATH/basecalled/
```

```
/group/pathogens/Bioinfo_Software/ont-guppy/bin/guppy_barcode --version
/group/pathogens/Bioinfo_Software/ont-guppy/bin/guppy_barcode --recursive --device cuda:all:100% --barcode_kits
RBK004 --num_extra_bases_trim 5 --min_score_barcode_front 70 --records_per_fastq 0 --compress_fastq --input_path
FULL_PATH /fast5_pass/guppy_basecalled/ --save_path FULL_PATH/guppy_basecalled/demultiplexed/
```

Determination of ST

- Fastq files- QC –not really needed as you have seen sequencing live, report

Protocol – 1 StringMLST <https://github.com/jordanlab>

2-step method

--buildDB

```
stringMLST.py --buildDB -c Path_to_file/config.txt -k 35 -P XF
```

--predict

```
stringMLST.py --predict -1 /path_to_fasta_or_fastq -s --prefix /path_to_StringMLSTdatabase -o /sampleST.txt
```

Protocol -2 Blast with a custom database of all alleles of 7 genes downloaded from pubMLST website

```
makeblastdb -in /path_to_fasta_file -dbtype nucl -title name_db -out name_db
```

```
seqtk seq -a fastq > fasta
```

```
blastn -db /Path_to_/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -  
query /path_to_fasta | grep "gene_name_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r > gene_name.txt
```


MLST amplicon seq of metagenomic samples

Host	Sample	Harper qPCR CT	cysG	gltT	holC	leuA	malF	nuoL	petC	ST	Subspecies
Grapevine	Vitis-B1-2	21.80	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B1-3	21.87	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-1	26.46	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-3	27.90	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-4	27.63	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-5	25.75	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-6	25.12	1	1	1	1	1	1	1	1	Xff
Grapevine	Vitis-B2-7	31.68	1	1	1	1	1	1	1	1	Xff
Oleander	Olea-B3-1	24.12	2	2	2	2	2	2	2	5	Xfs
Oleander	Olea-B4-2	24.69	2	2	2	2	2	2	2	5	Xfs

Average reading from samples	
Mean read length:	765.4
Mean read quality:	18.9
Median read length:	692
Median read quality:	15
Number of reads:	91,700
Read length N50:	736
Total bases:	70,184,875

Higher Sensitivity of Nanopore seq for MLST

Spiked concentration of Xf DNA in Olive		qPCR assay	LAMP assay	Illumina Miseq	Nanopore-MLST amplicon seq
		Harper et al 2010	Harper et al 2010 (Tp)	StringMLST	StringMLST
No dilution	240 pg/μl	21.90	9.9	ST	ST
10 ⁻¹	24 pg/μl	24.52	11.2	ST	ST
10 ⁻²	2.4 pg/μl	27.87	12.9	XF	ST
10 ⁻³	240 fg/μl	31.17	14.7	UD	ST
10 ⁻⁴	24 fg/μl	34.21	20.4	UD	ST
10 ⁻⁵	2.4 fg/μl	37.45	UD		ST
10 ⁻⁶	240 ag/μl	UD	UD		XF*
10 ⁻⁷	24 ag/μl	UD	UD		UD
10 ⁻⁸	2.4 ag/μl	UD	UD		UD

UD unidentified; * cut off 1000 reads

10 times more sensitive than qPCR

Thanks!

Tongda Li- AVR

MT17006 project team from VIC, NSW, QLd, WA
and NZ



Questions?

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Upcoming changes to ONT products

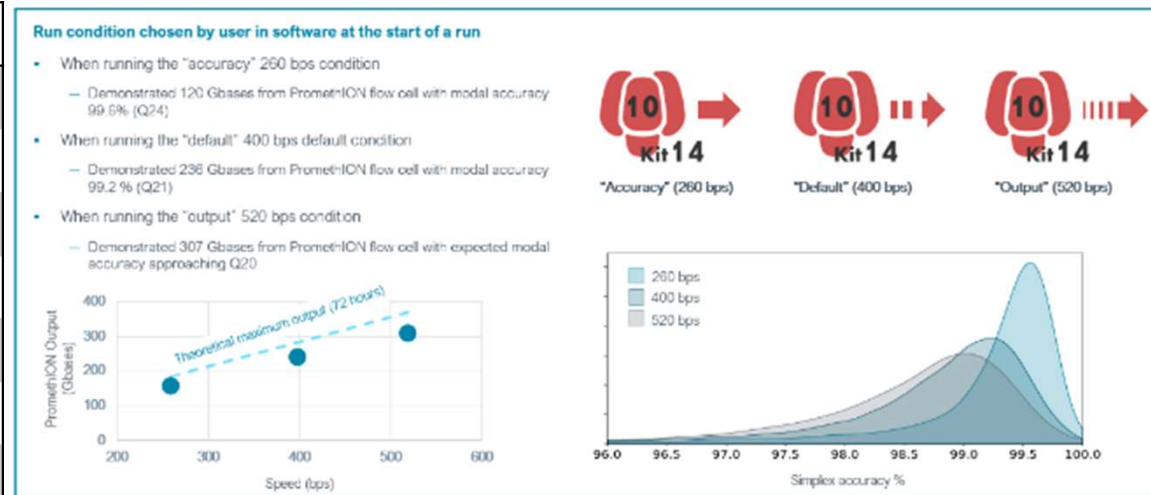
- V9 and V10 kits are scheduled to be discontinued starting from February
 - [Product update: kits scheduled to be discontinued \(nanoporetech.com\)](https://nanoporetech.com)
 - [The power of Q20+ chemistry \(nanoporetech.com\)](https://nanoporetech.com)

Flow cells			
Flow cell name	V14 product code	R10.4 flow cells to be replaced	R9.4.1 flow cells to be replaced
MinION Flow Cell	FLO-MIN114	FLO-MIN112	FLO-MIN106D
PromethION Flow Cell	FLO-PRO114M	FLO-PRO112	FLO-PRO002
Flongle Flow Cell	FLO-FLG114	N/A	FLO-FLG001

Nanopore Sequencing New Chemistry

R 9.4 flowcell → R 10.4 flowcell
K 9/10 Kits → K12/14 kits

Kit 14 Chemistry (Q20+)
LSK114
Used with R 10.4.1 flowcells
Raw read accuracy > 99%
Runs at ~250 or 400 or 520
Fuel fix adapter
Higher capture adapter
Duplex capable



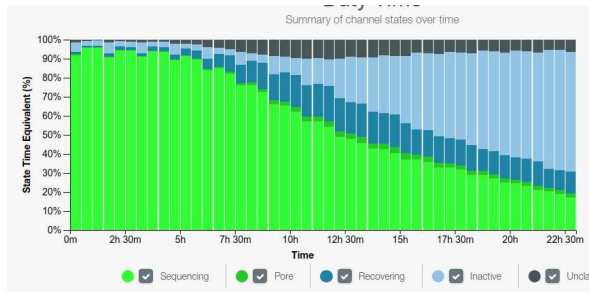
Different models are available for the base calling: fast, accurate and super accurate

Library preparation

- Quantify DNA- qubit ~ 400ng DNA
- Make equal quantification for all PCR products- pool for a sample
- Beads clean up and elute in 7.5 ul of V
- Rapid kit is transposon based, can't control equal DNA distribution for each barcode
- Use new chemistry is available to use Kit 14 and R10 flow cells for sequencing.
- Sequencing is controlled by MinKNOW version 22.10.7

If running many samples, do fast base calling and live demultiplexing to see the reads distribution.

Important tips



Never over load your flow cells, especially using amplicons



Loading the flow cell is tricky, introduction of any air bubble will kill the pores



Higher depth is important to deal with errors but with new chemistry, it may not required

PROTOCOL 1

stringMLST <https://github.com/jordanlab>

Installation with conda

```
conda create --name stringMLST
```

```
conda activate stringMLST
```

```
conda install stringMLST
```

to run it requires a database to be build using `–buildDB`

download both alleles and MLST profiles to build it. Make a txt file as given below and run `–buildDB`

loci]

leuA path_to_fasta/leuA.fa

petC path_to_fasta/petC.fa

malF path_to_fasta/malF.fa

cysG path_to_fasta/cysG.fa

holC path_to_fasta/holC.fa

nuoL path_to_fasta/nuoL.fa

glT path_to_fastap/glT.fa

Protocol 2- Blast

Download all the allele sequences in fasta files for each gene.

Make a database of the 7 MLST genes alleles.

```
makeblastdb -in /path_to_fasta_file -dbtype nucl -title name_db -out name_db
```

```
blastn -db /Path_to_/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -  
query /path_to_fasta | grep "gene_name_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r $> gene_name.txt
```

The above scripts will provide which allele is in abundance for a gene, repeat and grep each gene. Again, do Similar you can blast for rest of the MLST genes e.g petC as mentioned below

```
blastn -db /group/pathogens/plant_micro/projects/Xylella/Pragya/Minion/Xf_dilution/MLSTdatabase -num_threads 8 -  
task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -query path_to_fasta | grep "petc_" | awk '{print $1}' | sort -  
> petC.txt
```

Do so for all seven genes.