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RA1B-MR Diagnostics: Development and deployment of an *Austropuccinia psidii* biotype differential diagnostic test

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March 2024

Report for:

Biological Heritage

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Executive summary

RA1B-MR Diagnostics: Development and deployment of an *Austropuccinia psidii* biotype differential diagnostic test

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Five Loop Mediated Isothermal Amplification (LAMP) assays, designed by USA-based colleagues to specifically diagnose four of the genetic clusters (clades) of *Austropuccinia psidii* (Pandemic (two assays), Eucalyptus, Guava, South African) were tested at The New Zealand Institute for Plant and Food Research Limited (PFR), Lincoln using a plasmid with an insert containing the differential sequence targets. The LAMP assay was also tested against 12 genomic DNA preparations from fungal spores collected from infected plants in Aotearoa-New Zealand.

The two assays targeting the Pandemic and the assays targeting the Eucalyptus and Guava clades all generated a positive result when 10^{12} copies of the positive control target sequence were present. The assay targeting the South Africa clade sequence failed to produce a positive result.

A dilution series (10^{12}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2} and 10^{1} copies) of the positive control plasmid yielded inconsistent results when assayed. The Eucalyptus clade assay was positive at a very low target number of 10^{1} copies, the South African assay was negative at all dilutions (with a putative false positive at 10^{2} copies), the Guava assay was positive down to 10^{5} copies, whilst the two Pandemic assays were only positive at 10^{12} and 10^{6} copies. The putative false positives that were observed are likely a consequence of the pH sensitivity of the dye in the LAMP assay. There were no false positives in the negative controls.

When the assays were assessed against 12 *A. psidii* genomic DNA preparations from fungal spores collected from infected plants in Aotearoa-New Zealand, only the Pandemic-AUS 1.77 assay produced clear positive results from four of the 12 DNA extracts whilst the Pandemic-PanAus produced one. The Eucalyptus assay also produced a positive result from one sample; at this point this is presumed to be a false positive similar to those found with the control plasmid experiments.

Following discussions with our Australian colleagues at the Queensland Department of Agriculture and Fisheries, a set of assays targeting the mating compatibility locus (HD1 region) in the fungal genome were designed, as previous phylogenetic analysis of this region had found sufficient variability that was suitable for targeted differential diagnostics. A Recombinase Polymerase Assay (RPA), that included a control for the presence of fungal DNA, was developed that is capable of distinguishing between the four *A. psidii* biotypes in the laboratory. Persistent logistical issues with importing reagents for this assay prevented successful field demonstration.

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1 Introduction

Austropuccinia psidii, the pathogen that causes the disease myrtle rust, shows genetic diversity, and to date nine distinct genetic clusters (C1 to C9) have been identified (Stewart et al. 2017). Two clusters, C1 and C4, comprise the Pandemic biotype, the variant of *A. psidii* found in Aotearoa-New Zealand, Australia and New Caledonia. Two other biotypes have been identified: a Eucalypt biotype (comprised of the C2 and C3 clusters) and a Guava biotype (C6 cluster). The unique genotype found on multiple myrtaceous hosts in South Africa was not included in this assessment, but it is distinct from the Pandemic biotype. Thus, there are at least seven genetic variants of *A. psidii* (two biotypes, four genetic clusters and the South African genotype) that are genetically distinct from the Pandemic biotype found in Aotearoa-New Zealand. The threat of these other genetic variants to the native and introduced myrtaceous species in this country is unclear, however, in general the introduction of additional genetic diversity of an invasive pest or pathogen complicates response management (e.g. breakdown of known host resistance). We have already established there is limited (mānuka, kānuka), very limited (pōhutukawa) or no (ramarama, rohutu) resistance in taonga species in Aotearoa-New Zealand (Smith et al. 2020). The risk to Aotearoa-New Zealand myrtle species is already significant and likely to increase should another *A. psidii* biotype or genetic variant establish in Aotearoa-New Zealand.

2 Development of assays to differentiate *A. psidii* clades

Clades in this context is used as a descriptor for four of the seven genetic variants of *A. psidii* (two biotypes, four genetic clusters and the South African genotype). Five Loop Mediated Isothermal Amplification (LAMP) assays were designed by Jorge Ibarra Caballero, University of Colorado using *A. psidii* genomic data available on the National Center for Biotechnology Information (NCBI) database targeting four of the clades (Table 1). These assays were initially assessed in Aotearoa-New Zealand using the positive control sequences and then against *A. psidii* genomic DNA extracted from spores collected from infected plants in Aotearoa-New Zealand (Table 2) using the protocol described in Appendix 1.

Table 1. *Austropuccinia psidii* diagnostic assay clade targets.

Reference	Clade
PanAus	Pandemic
AUS1.77	Pandemic
EUC	Eucalyptus
PGS (PGSC)	Guava
SA1.77	South Africa

A pUC57 plasmid insert (KpnI/BamHI) sequence positive control containing all five target sequences was designed by Falk Kalamorz (Figure 1) and commercially prepared by Genscript Biotech, Singapore.

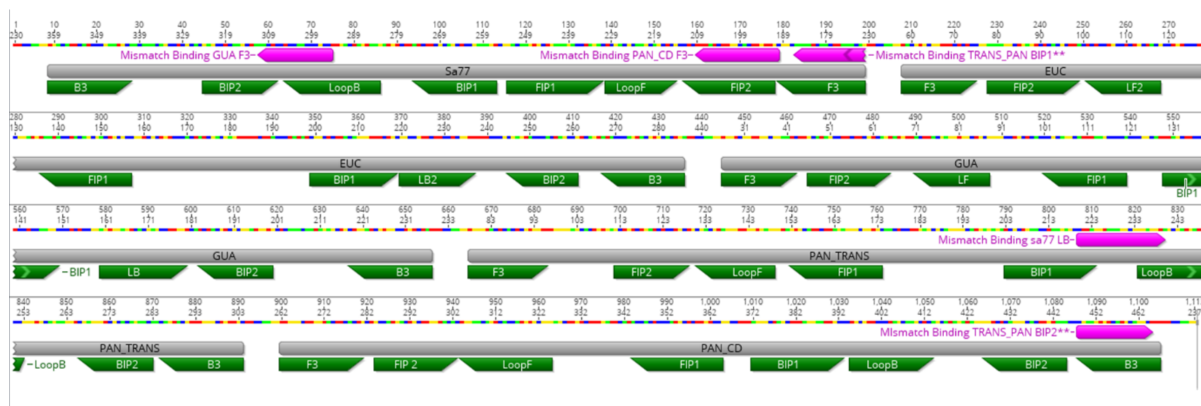

Figure 1. The plasmid positive control region for the *Austropuccinia psidii* Loop Mediated Isothermal Amplification (LAMP) assays. Target amplicons are shown in grey. Primer binding sites are shown as green arrows. Mismatch binding sites (predicted by Geneious 10.0.9) between different assays are shown as purple arrows. The sequence and orientation of target amplicons was optimised to prevent putative unspecific secondary reactions.

Table 2. Identification reference and source of genomic DNA extracted from Aotearoa-New Zealand isolates of *Austropuccinia psidii.*

Identification number	Plant host species
175_LB	*Lophomyrtus bullata*
245_LB	*Lophomyrtus bullata*
370_LB	*Lophomyrtus bullata*
95_LO	*Lophomyrtus obcordata*
179_LO	*Lophomyrtus obcordata*
14_MD	*Metrosideros diffusa*
18_MD	*Metrosideros diffusa*
60_MD	*Metrosideros diffusa*
210_ME	*Metrosideros excelsa*
230_ME	*Metrosideros excelsa*
289_ME	*Metrosideros excelsa*
84_SJ	*Syzygium jambos*

3 Results

3.1 Initial evaluation against positive control (the plasmid)

The assays targeting the Pandemic (two assays), Eucalyptus and Guava clades all generated a positive (solution colour changes from red to yellow) result when 10^{12} copies of the positive control target sequence were present. The assay targeting the South Africa clade failed to produce a positive result after 30 min (Figure 2).

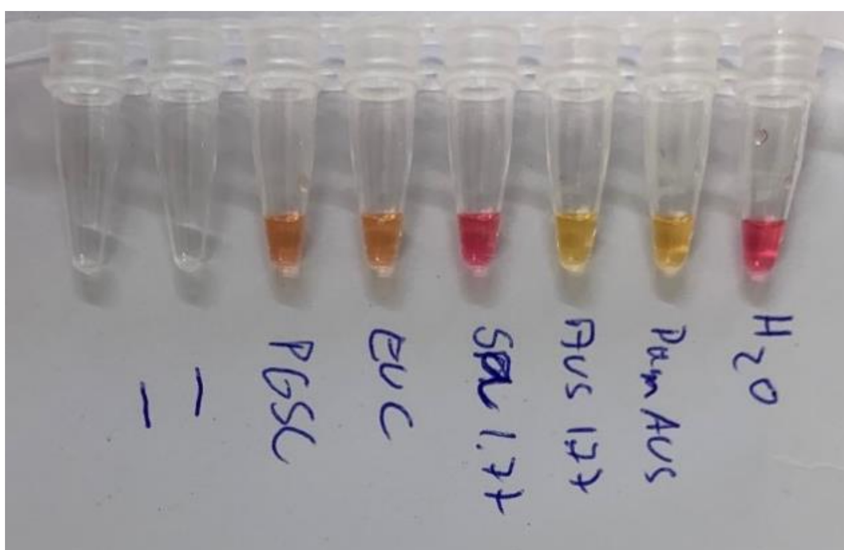

Figure 2. Result of Loop Mediated Isothermal Amplification (LAMP) assays with 10^{12} copy numbers of target sequence. The assays were performed following the manufacturer's instructions and Jorge Ibarra Caballero's protocol. Template DNA was added to the master mix. The two tubes containing clear liquid (left) are reagent negative controls (no primer nor template). A colour change from pink to yellow is a positive.

3.2 Time course of detection

The progression of the reaction over time was observed. A dilution series of the plasmid positive control was prepared to produce copy numbers of 10^{12}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2} and 10^{1}. The reaction time is the cumulative time the reaction was exposed to a temperature of 65°C, i.e. a first step of 30 min at 65°C was performed followed by a decrease of temperature to 12°C and observation/documentation of the samples, followed by a series of 10 min incubation at 65°C, followed by a decrease of temperature to 12°C and further observation/documentation of the same sample.

After 30 min, a copy number of 10^{12} led to a positive result for the Eucalyptus and Guava clades, while copy numbers of 10^{12} and 10^{6} both produced positive results for the two Pandemic clade assays. The South African clade assay failed to produce a positive result. Instead, an immediate false positive in sample 10^{2} was observed, which changed colour to bright yellow immediately after addition of the master mix. The reason for this false positive was not determined (Figure 3 and 4, Middle panel).

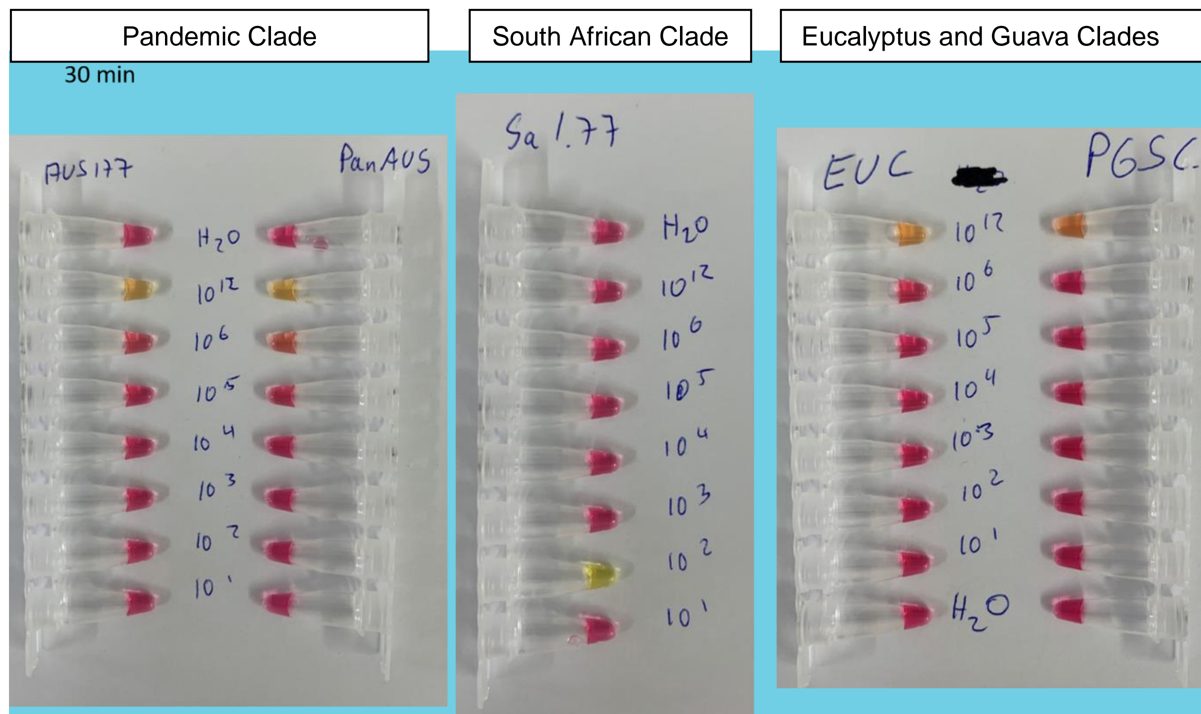

Figure 3. Loop Mediated Isothermal Amplification (LAMP) assays with different target copy numbers of the positive control plasmid after 30 min. Positive detection is indicated by a change from red to yellowish shade. A false positive is evident in the 10^2 assay in the South African clade series.

After 50 min, all the Eucalyptus clade reactions containing the plasmid target sequence were positive (yellow), indicating that a loop-mediated reaction took place as expected. The Pandemic clade assays were positive down to 10^5 target sequences, and the Guava clade assay only produced a positive result for assays including 10^{12} and 10^6 target sequences. This result suggests a time-dependent linear amplification was occurring rather than a loop-mediated amplification. No consistent positive result was observed in the South African clade assays (Figure 4).

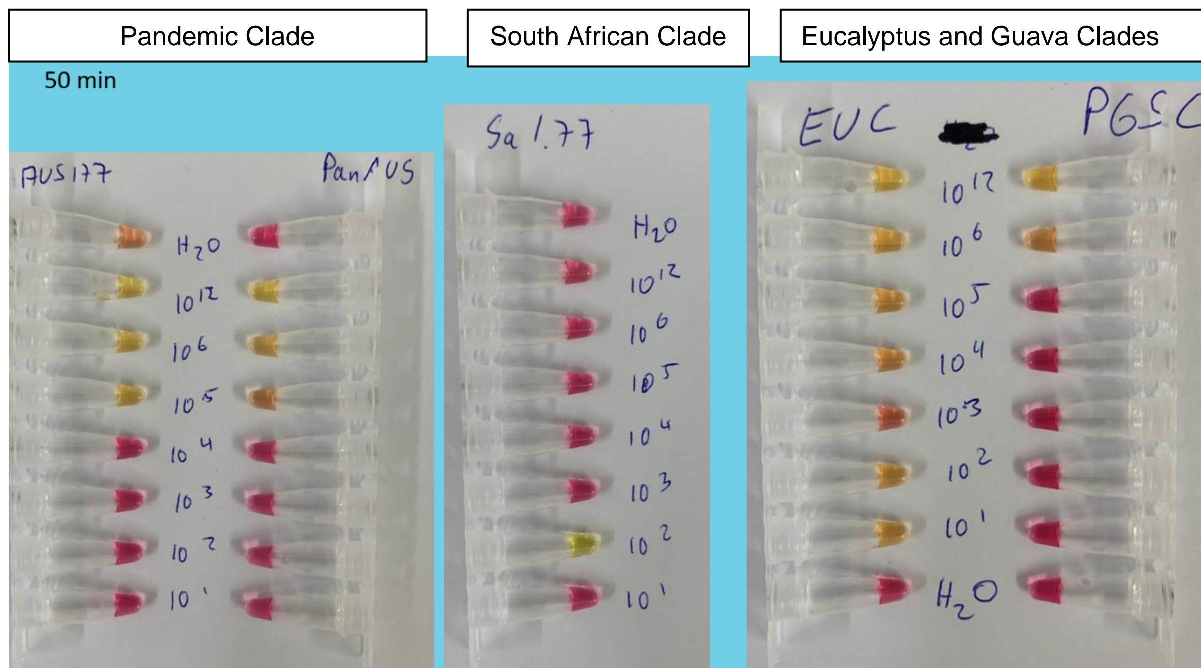

Figure 4. Loop Mediated Isothermal Amplification (LAMP) assays with different target copy numbers of the positive control plasmid after 50 min. Positive detection is a indicated by a change from red to yellowish shade. A false positive is evident in the 10^2 assay in the South African clade series.

At the end point of 90 min most of the reactions showed a strong positive (yellow), including several of the no template controls. This may be caused by nonspecific breakdown of assay components leading to a sufficient pH change for the indicator switch, or the creation of small amplifiable constructs based on primer interactions. One of Pandemic clade assays (PanAUS) yielded results as expected. The South Africa clade assay showed strong nonspecific reactions, with pH changes apparently unrelated to the amount of target sequence present (Figure 5).

3.3 Initial assessment of DNA from Aotearoa-New Zealand field samples of *A. psidii*

The Pandemic-AUS 1.77 assay produced clear positive results from four of the 12 DNA extracts from *A. psidii* found in Aotearoa-New Zealand, whilst the Pandemic-PanAus produced one (Figure 6). The Eucalytpus assay also produced a positive result from one sample, however this is likely to be a false positive similar to those found with the control plasmid experiments.

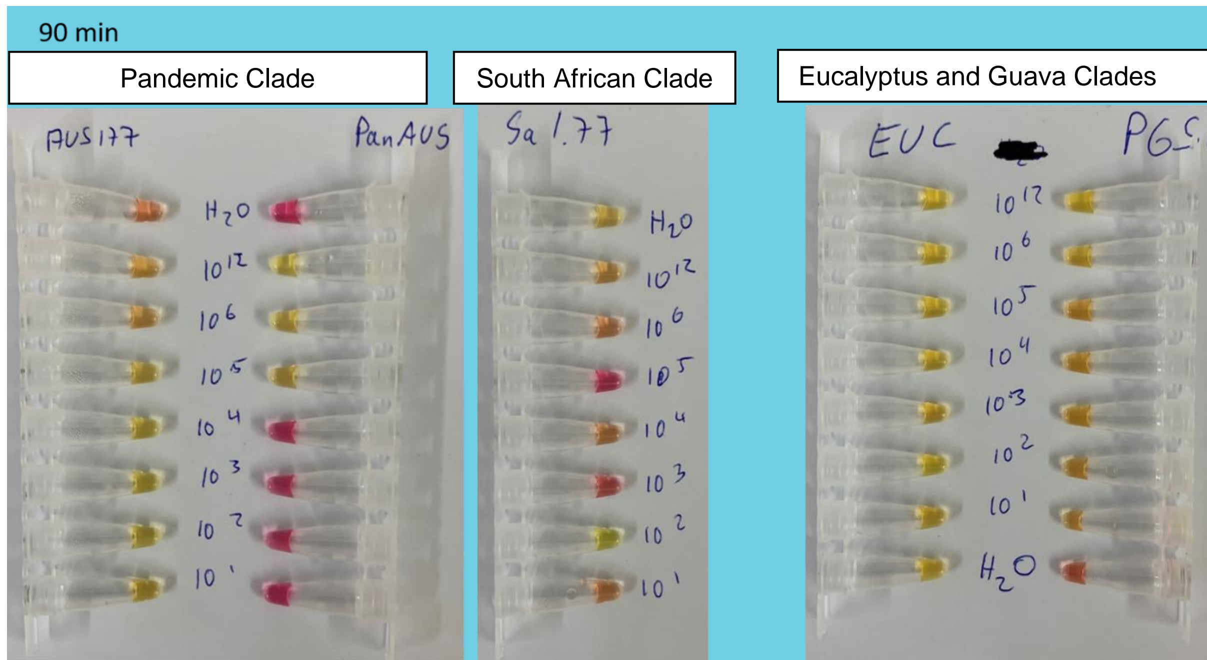

Figure 5. Loop Mediated Isothermal Amplification (LAMP) assays with different target copy numbers of the positive control plasmid after 50 min. Positive detection is a indicated by a change from red to a shade of yellow. False positives are evident in the South African clade series.

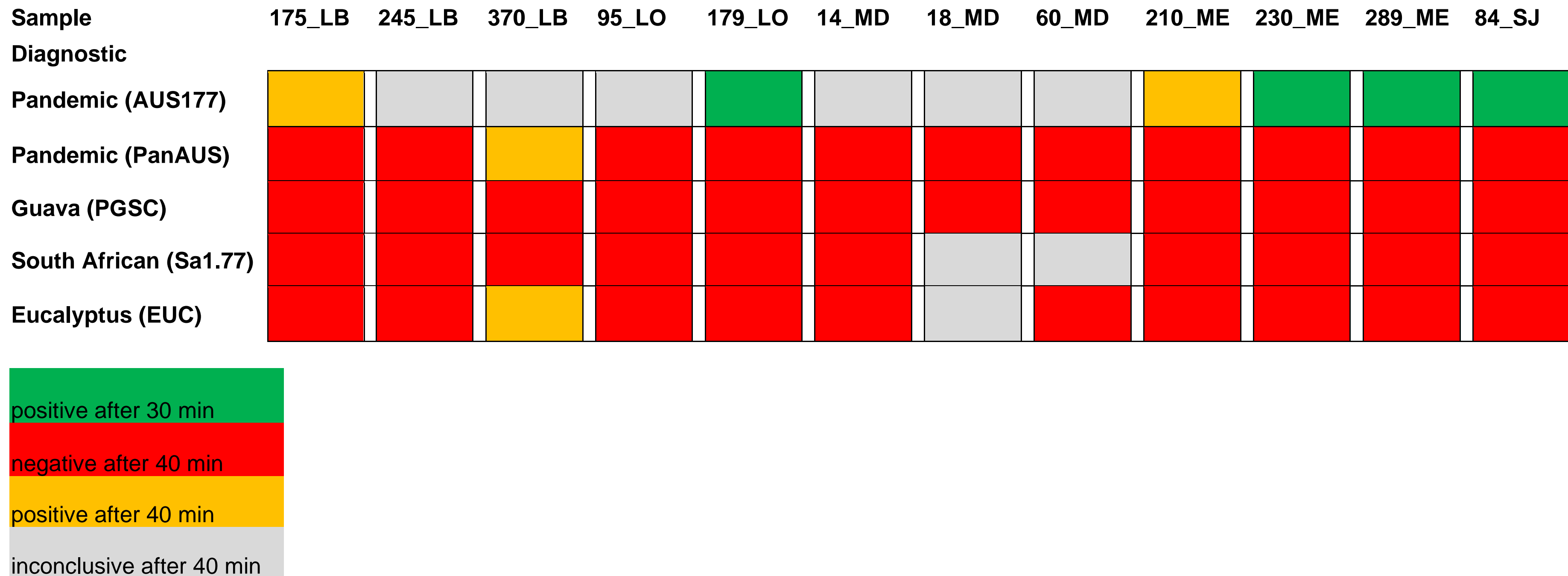

Figure 6. Summary of the first assessment of five Loop Mediated Isothermal Amplification (LAMP) assays with *Austropuccinia psidii* genomic DNA preparations from spores collected from 12 infected plants in Aotearoa-New Zealand. LB, *Lophomyrtus bullata;* LO, *Lophomyrtus obcordata;* MD, *Metrosideros diffusa;* ME, *Metrosideros excelsa;* SJ, *Syzygium jambos.* “Inconclusive” indicates an intermediate colour change (“orange”), as per Figure 2.

4 In-Field Recombinase Polymerase Amplification (RPA) diagnostic for the Pandemic strain of *A. psidii*

4.1 Introduction

Due to the issues encountered with the LAMP assay described in the Results section, an alternative assay based on the also field-deployable Recombinase Polymerase Amplification (RPA) technology was developed. RPA technology requires minimal equipment and results are quick and easy to assess through end-point visualisation of reaction products via SYBR Green or a similar dye. Additionally, RPA assays can be multiplexed through the inclusion of fluorescent probes, albeit this approach will require laboratory-based equipment for signal analysis.

4.2 Diagnostic region selection

Sequence information was provided by Louise Shuey (Department of Agriculture and Fisheries, Queensland, Australia). Their previous phylogenetic analysis of the HD1 region included representatives of three of the *A. psidii* biotypes (Pandemic, Brazilian/Eucalyptus and South African) and they found sufficient variance in that region to make it suitable for targeted differential diagnostics.

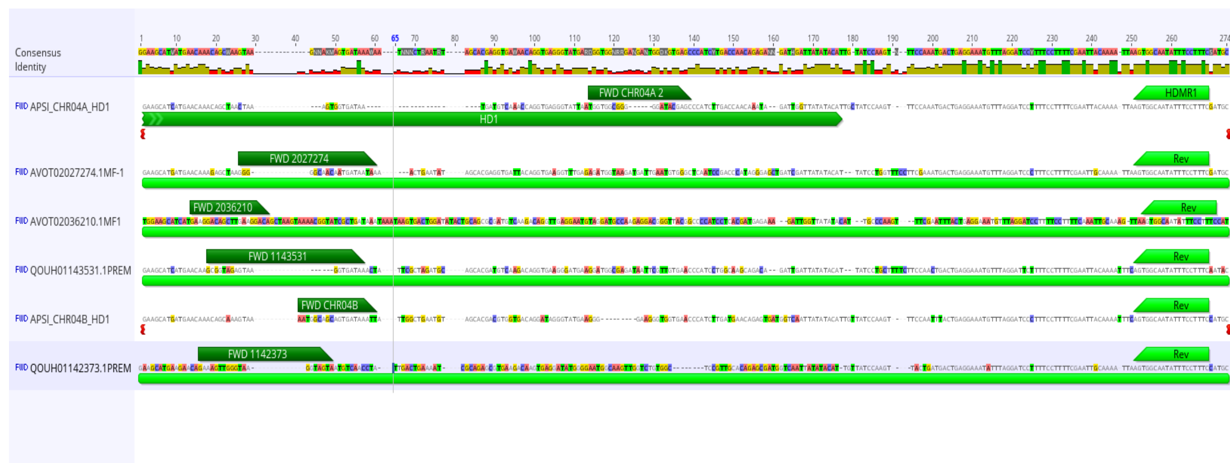

Figure 7. Alignment of HD1 regions of the *Austropuccinia psidii* biotypes covered in the Recombinase Polymerase Amplification (RPA) assay. Primer binding sites are shown as green arrows. CHR04 – Pandemic biotype. AVOT – Brazilian/Eucalyptus biotype. QUOH – South African biotype. Primers were selected in low homology regions and verified to not bind the target region of other biotypes with up to four mismatches (predicted by Geneious 10.0.9).

Our RPA assay based isothermal amplification diagnostic approach uses the universal HDMR1 reverse primer (Feng et. al 2024) in combination with individual clade-specific forward primers.

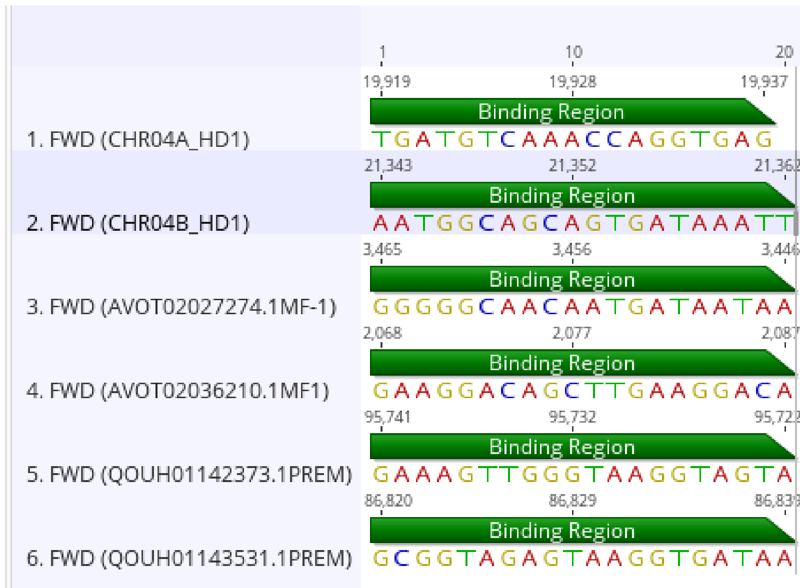

Figure 8. Forward primers for amplification of HDR regions of the *Austropuccinia psidii* biotypes covered in the Recombinase Polymerase Amplification (RPA) assay. CHR04 – Pandemic biotype. AVOT – Brazilian/Eucalyptus biotype. QUOH – South African biotype.

4.3 A control for the presence of fungal DNA was developed using previously published ITS primers (Toju et al. 2012). Validation of targets via polymerase chain reaction (PCR)

Firstly, the specificity of the forward primers for their respective biotype were established through traditional PCR (Figure 9). Additionally, it was confirmed that the ITS primers can be used to amplify *A. psidii* DNA. In this assay, the primer pair 2036210/HDMR1 produced an unexpected product when 1142373 was used as template. Therefore, we recommend the use of forward primer 2027274 to identify the Brazilian/Eucalyptus biotype.

A control for the presence of fungal DNA was developed using previously published ITS primers (Toju et al. 2012, Figure 10).

Figure 9. Validation of target specificity of primers for Recombinase Polymerase Amplification (RPA) assays. Left side above parenthesis: primer pair used and expected product size. Right side above parenthesis: template DNA. CHR04A & B – Pandemic biotype. 20227274 & 2036210 – Brazilian/Eucalyptus biotype. 1142373 & 1143521 – South African biotype. Blue background indicates template DNA that produced an expected band. Green background indicates template DNA that produced a not expected product. Visualisation of polymerase chain reaction (PCR) products after separation on 1% SBS agarose gel under UV light.

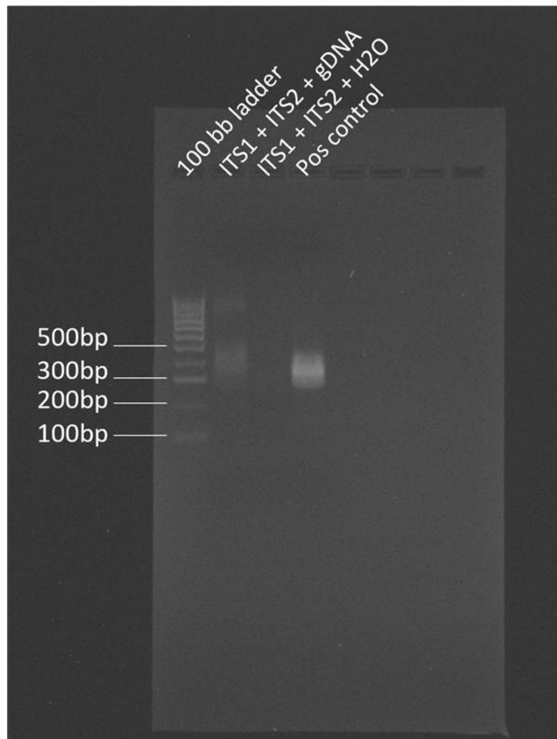

Figure 10. Validation of ITS control. Details of primers and reaction are described in Toju et al. (2012) using genomic DNA from the Pandemic clade of *Austropuccinia psidii*. Visualisation of polymerase chain reaction (PCR) products after separation on 1% SBS agarose gel under UV light.

4.4 Recombinase Polymerase Assay

With the knowledge that the primer combinations are specific in PCR, and the economic decision that any field deployable test should primarily be able to tell if a sample is the Pandemic biotype currently present in Aotearoa or not, only the CHR04A forward primer was validated in the RPA assay system (Figure 11).

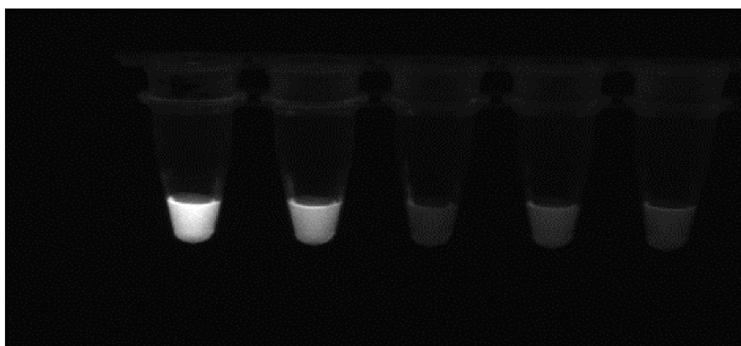

Figure 11. Validation of target specificity of CHR04A primer for Recombinase Polymerase Amplification with the universal HDMR1 reverse primer in (RPA) assays. Left to right: kit positive control, CHR04 (Pandemic) template, AVOT (Brazilian/Eucalyptus) template, QUO (South African) template, negative control. Visualisation after addition of 1 µl STBR Green solution under UV light.

4.5 In-field and cross-laboratory validation

In-field validation was attempted using infected material in situ. It was not successful, and the overall failure of the RPA reactions, including positive controls, implies that either kit components were damaged during transfer or that the incubation conditions at an ambient temperature of 18°C did not allow the amplification reaction to occur.

Subsequent attempts to replicate the RPA assay at SCION with material provided by The New Zealand Institute for Plant and Food Research Limited (PFR) also failed, which in our opinion indicates a general issue with the Twist Liquid Basic kit. We observed significantly shorter shelf life of components than the 12 months recommended by the manufacturer, and both of the kits we purchased in 2023 were held at the border for several weeks due to biosecurity concerns despite our best efforts to avoid such issues. This border transition duration may have contributed to limiting the integrity of the kit components.

One way to avoid such issues in the future is to switch to the Twist Amp® lyophilised Basic kit which, being lyophilized, should have a longer shelf life, but this would also cause increased preparation and make assays more difficult in the field with higher costs.

5 Summary

An RPA assay, that includes a control for the presence of fungal DNA, has been developed that is capable of distinguishing between four of the *A. psidii* biotypes. This assay will give one of the following results:

1. For a plant that is either symptomatic for the myrtle rust (i.e. purple lesions/patches on leaves and/or yellow spores) or asymptomatic, a positive result for both fungal DNA and the Pandemic biotype would indicate that the sample is infected with the *A. psidii* Pandemic biotype.
2. A positive result for fungal DNA and a negative result for the *A. psidii* Pandemic biotype. This result has two different potential causes.
    a. If the plant is showing myrtle rust symptoms it is potentially infected with another biotype of *A. psidii* that the assay does not detect. The Ministry for Primary Industries (MPI) needs to be immediately advised of this finding.
    b. If the plant is not showing myrtle rust symptoms, then the detection of fungal DNA is likely that of saprophytic fungi. Monitoring and re-testing of this plant is recommended.
3. A negative result for both fungal DNA and the Pandemic biotype. This indicates either an issue with the DNA extraction (no/insufficient template) or, albeit rather unlikely, a misidentification of symptoms.

6 References

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Toju H, Tanabe AS, Yamamoto S, Sato H 2012. High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. Plos ONE 7(7): e40863. https://doi.org/10.1371/journal.pone.0040863.

Appendix 1. Chelex extraction protocol for extraction of genomic DNA from *Austropuccinia psidii* spores

Equipment

- Heat block or bath @ 95°C
- Vortex
- Centrifuge (set up for 1.5 ml tubes)
- Pipette and tips – 200µl size
- 1.5 ml tubes (2 per sample – label tubes before starting)
- 5% Chelex solution in TE buffer (5 g/100 ml)

Method

- Vortex the Chelex solution (important that the resin beads are suspended in solution and not all at the bottom of the tube).
- Add 200 µl 5% Chelex solution to each sample (you could pick single pustules and put them straight into this solution if you are going to proceed with the extraction on the same day).
- Heat tubes for 8 min at 95°C.
- Vortex, heat again for 7 min at 95°C.
- Spin in centrifuge at 12,000 g for 1.5 min at room temperature.

Transfer supernatant into clean 1.5 ml tube, taking care to avoid transferring any chelex resin. Discard the tubes with the resin left in the bottom. The new tube contains your DNA extraction.

Appendix 2. Protocol for Recombinase Polymerase Amplification (RPA) assay to identify the Pandemic strain of *Austropuccinia psidii* currently present in New Zealand

Background:

This assay is designed to reliably and specifically identify the Pandemic strain of *A. psidii*. It consists of a test reaction, a DNA quality control reaction, an internal positive control provided by the manufacturer and a no template control.

Material:

TwistDX Liquid Basic kit

CHR04A primers for test reaction

	FWD	TGGTGGCGGGGGATACGAGC
	REV	GAAAGGAAATATTGCCACT

ITS primers for DNA quality control reaction

	FWD	TAGAGGAAGTAAAAGTCGTAA
	REV	TTYRCTRCGTTCTTCATC

Material for DNA extraction: sterile 1.7 ml Eppendorf tubes with each 0.5 ml and 1 ml of sterile 1 M Tris-HCl per sample
sterile pestles

SYBR Green Nucleic Acid Stain (Caution: this is an intercalating agent and has carcinogenic potential)

Set up:

Prepare an appropriate amount of master mix according to the manufacturer's instructions and mix with the appropriate primers in the test tubes (without template and MgOAc). In our experience, the reaction volume can be reduced to 25 µl. We recommend setting up two sets of test reaction, DNA quality reaction, internal positive and no template control (using CHR04A primers) in one 8-tube, 0.25 ml tube strip.

Keep cool until use.

DNA extraction:

We do not expect this method to be able to extract DNA from spores. Therefore, plant material with actively growing *A. psidii* hyphae should be used.

- Add a piece of infected leaf about 1 cm x 2 cm to 0.5 ml sterile Tris-HCl and crush thoroughly with a sterile pestle.
- Transfer 50 µl of the extract into a fresh tube of 1 ml Tris-HCL. Keep tube with crushed leaf.
- Use 1 µl of the dilution as template for the test reaction and the DNA quality reaction.

RPA assay:

- Let tubes warm to ambient temperature.
- Refer to manufacturer's instructions for further details on procedure.
- Add 1 µl sample to the test reaction and the DNA quality control reaction.
- Add 1 µl of template to the internal control reaction.
- Add MgOAc to all tubes to start reaction.
- Incubate reactions for 30 min at 45°C (for example in a portable dry bath), or 1 h at an ambient temperature.
- [Note: The assay is not suitable for incubation temperatures below 25°C – if ambient temperatures are low and no heating device is available, consider incubation in a car with the heating on or a similar set-up.]
- Add 1 µl of undiluted SYBR Green Nucleic Acid Stain to the tubes.

Interpretation of results:

The tubes should show either a reddish or greenish colour. Green indicates amplification of DNA, i.e. a positive result.

- The internal positive control should be green.
- The no template control should be orange.
- The DNA quality control should be green.
- The test reaction can be either green or orange.

If the controls are correct, and the test reaction is green, it indicates that the tested plant material contains the Pandemic strain of *A. psidii*.

If the controls are correct, and the test reaction is red, two scenarios are possible:

A. the tested material contains fungal DNA that is not *A. psidii*

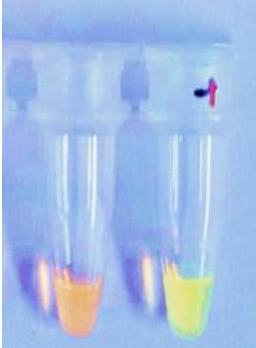
B. the tested material contains *A. psidii* that is not the Pandemic strain.

If such a result is obtained, the associated sample tube with the crushed leaf, and ideally additional infected material, should be collected and tested in greater detail in the laboratory.

Example of taking leaf sample:

Comparison before/after crushing:

Example for negative (orange) and positive (green) SYBR stain:



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