Real-Time PCR Assays for the Detection of *Puccinia psidii*

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Abstract

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Puccinia psidii (Myrtle rust) is an emerging pathogen that has a wide host range in the Myrtaceae family; it continues to show an increase in geographic range and is considered to be a significant threat to Myrtaceae plants worldwide. In this study, we describe the development and validation of three novel real-time polymerase reaction (qPCR) assays using ribosomal DNA and β-tubulin gene sequences to detect *P. psidii*. All qPCR assays were able to detect *P. psidii* DNA extracted from urediniospores and from infected plants, including asymptomatic leaf tissues. Depending on the gene target, qPCR was able to detect down to 0.011 pg of *P. psidii* DNA. The most optimum qPCR assay was shown to be highly specific, repeatable, and reproducible following testing using different qPCR reagents and real-time PCR platforms in different laboratories. In addition, a duplex qPCR assay was developed to allow coamplification of the cytochrome oxidase gene from host plants for use as an internal PCR control. The most optimum qPCR assay proved to be faster and more sensitive than the previously published nested PCR assay and will be particularly useful for high-throughput testing and to detect *P. psidii* at the early stages of infection, before the development of sporulating rust pustules.

Puccinia psidii G. Winter is a biotrophic fungal pathogen causing a serious rust disease (also known as guava rust, eucalyptus rust, myrtle rust, or ohia rust) on a wide range of plant species in several genera of Myrtaceae (Langrell et al. 2008). *P. psidii* was first reported in Brazil in 1884 (Winter 1884), and then spread to other South and Central American countries. Later, spread of *P. psidii* has been reported from different states in the United States, including California, Florida (Rayachhetry et al. 1997), and Hawaii (Uchida et al. 2006). Recently, this pathogen has been reported from different countries on different continents, including Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2010), China (Zhuang and Wei 2011), South Africa (Roux et al. 2013), and New Caledonia (Giblin 2013). The recent emergence of this pathogen and its vigorous spread threatens species in the Myrtaceae family worldwide, including endemic species in many countries (Carnegie and Lidbetter 2012; Coutinho et al. 1998; Glen et al. 2007; Tommerup et al. 2003). New Zealand is free from *P. psidii* but it is feared that this rust may arrive in the near future because rust spores have been reported to travel long distances on wind currents. For example, cereal and poplar rusts were shown to be introduced into New Zealand from Australia by wind currents (Viljanen-Rollinson and Cromey 2002; Wilkinson and Spiers 1976).

Unlike most rust species, *P. psidii* has a wide host range (Carnegie and Lidbetter 2012). Since it was found in New South Wales, Australia in early 2010, the host range increased from 100 to more than 400 species (Giblin and Carnegie 2014; Simpson et al. 2006), causing a significant impact on the environment, economy, and community (Carnegie and Cooper 2011). *P. psidii* has caused significant economic impacts on *Eucalyptus* (Furtado and Marino 2003) and guava (Martins et al. 2011) plantations in South America, where outbreaks have resulted in 50 to 80% loss of production. Long-term impacts are likely to affect many industries such as nursery and garden, cut flower and foliage, forestry and timber, honey and pollination, bush foods and medicines, and revegetation and tourism. Like many other rust fungi, *P. psidii* is easily spread by dissemination of infectious urediniospores by wind, rain, and insects. Since the first detection of *P. psidii* in Australia, the fungus has spread rapidly along the eastern coast, likely due to the large number of airborne spores produced (Carnegie et al. 2010). Once *P. psidii* has been introduced and established, it is very difficult to contain and to prevent further spread. The long-distance spread of this fungus is likely to occur via urediniospores on imported nursery stocks or contaminated clothing. Conventional diagnosis of *P. psidii* relies on symptom observations, host identification, and microscopic examination of sporulating rust pustules and spores. However, it is difficult to identify myrtle rust based on early symptoms because the characteristic rust pustules may be absent. Moreover, *P. psidii* can cause a diverse range of early symptoms depending on host susceptibility and environmental conditions (Glen et al. 2007); therefore, morphological identification can be difficult and time consuming because it requires specialized expertise. Because any delay in pathogen identification may reduce the probability of successful containment or eradication, it is important to have alternative detection methods.

Identification of plant pathogens using molecular methods offers several advantages over traditional methods of diagnosis. Polymerase chain reaction (PCR)-based detection methods have made a significant contribution to eliminate some of the problems associated with the detection, control, and containment of plant pathogens (Henson and French 1993). The internal transcribed spacer (ITS) regions within the nuclear ribosome-encoding gene (rDNA) have been widely used in fungal taxonomy. including several species in genus *Puccinia* Pers. (Anikster et al. 2004; Kropp et al. 1997; Zambino and Szabo 1993). Furthermore, the rDNA gene is the most commonly sequenced for rust species to study their genetic relationships in rust fungi (Aime 2006). In addition, the high copy number of ITS regions in the fungal genome (Ganley and Kobayashi 2007) enables sensitive PCR amplification.

There is only one published PCR assay to detect *P. psidii*, where the ITS region was used to design a species-specific nested PCR assay (Langrell et al. 2008). This nested PCR assay required two rounds of PCR amplifications and gel electrophoresis to confirm the identity of *P. psidii*. The nested PCR approach can take up to 9 h following receipt of suspect infected tissue. An alternative to conventional PCR detection is the use of real-time PCR (qPCR), which

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allows accurate detection and quantification of plant pathogens (Capote et al. 2012). In addition, qPCR is highly sensitive and faster than conventional PCR, enabling the use of qPCR for high-throughput detection of plant pathogens.

Currently, there is no qPCR assay available for *P. psidii*. Accordingly, the objectives of this study were the development and validation of novel qPCR primers and hydrolysis probes for sensitive and fast detection of *P. psidii* in plant tissue.

Materials and Methods

***P. psidii* DNA extraction and infected plant specimens.** The qPCR assays were developed using DNA extracted from urediniospores of *P. psidii* collected from infected plants in Brazil, Hawaii, and Australia (Table 1). Further testing and validation of the qPCR assays were then conducted on *P. psidii*-infected leaves collected in Australia and from herbarium specimens obtained from New Caledonia (Table 1).

Two DNA extraction methods were used to extract DNA from *P. psidii*-infected plant tissues. The DNeasy Plant Mini kit (Qiagen) method was used to extract DNA from field-collected, *P. psidii*-infected leaf tissues of *Rhodomyrtus psidioides* (native guava) and *Rhodamnia rubescens* (scrub turpentine). Leaves with different levels of *P. psidii* infection ranging from nonsymptomatic to heavily infected were used to extract DNA to test the detection sensitivity of the qPCR assays (Fig. 1). Four leaf tissue sections (approximately 5 by 5 mm, containing rust pustules) were taken from an infected leaf and added into a 2-ml screw-capped tube containing a single chromo steel bead (3.2 mm in diameter). The samples were macerated for 2 min using a TissueLyser II (Qiagen) and then 600 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer (2.0% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl [pH 8.0], and 1.0% polyvinylpyrolidone) was added, mixed by inversion several times, and incubated at 65°C for 25 min, with occasional shaking for 20 s at 2-min intervals using a Thermomixer (Eppendorf). The samples were centrifuged at 13,000 rpm for 3 min, and 500 µl of supernatant was transferred to a new tube and purified according to the manufacturer's instructions.

An automated KingFisher (Thermo Scientific) nucleic acid extraction system with the InviMag plant DNA mini kit (Invitek) was also used to extract DNA from myrtle-rust-infected herbarium materials of *Syzygium jambos* (rose apple) and *Eugenia gocognei* (eugenia). Approximately 300 mg of leaf tissue with rust pustules was placed in an extraction bag (Bioreba) with 3 ml of CTAB lysis buffer. An automatic Homex grinder (Lenze) was used to grind the leaf tissue completely. Approximately 1.5 ml of the homogenized sample was incubated at 65°C for 25 min with occasional shaking for 20 s at 2-min intervals using a Thermomixer. Subsequently, the samples were centrifuged at 13,000 rpm for 3 min, and 420 µl of supernatant was used to perform DNA extraction using the KingFisher system with InviMag plant DNA mini kit (Invitek), according to the manufacturer's instructions. This method was also used to extract DNA from healthy Myrtaceae plant leaves (Table 2). Prior to qPCR tests, the concentration of all *P. psidii* DNA samples and all DNA extractions from infected samples were quantified using a NanoDrop ND1000 spectrophotometer (Thermo Scientific). The DNA samples were stored at −80°C until use for PCR applications.

Design of species-specific primers and TaqMan probes. In total, 46 complete ITS *P. psidii* sequences and four β-tubulin gene sequences of *P. psidii* were retrieved from GenBank (National Center for Biotechnology Information) and analyzed using Geneious software (Geneious Pro 5.5.6; Biomatters Ltd). Multiple forward and reverse primers and hydrolysis probes (TaqMan) were designed for

Fig. 1. *Rhodomyrtus psidioides* leaves with different levels of *Puccinia psidii* infection used for DNA extractions.

Table 1. Isolates of *Puccinia* spp. used in this study

Species	voucher	Host	DNA from	Origin
Puccinia psidii	UFV-2	*Eucalyptus* sp.	Urediniospores	Brazil
P. psidii	SUZ-14	*Syzygium jambos*	Urediniospores	Brazil
P. psidii	RJ-9	*Psidium guajava*	Urediniospores	Brazil
P. psidii	HW-1	*S. jambos*	Urediniospores	Hawaii
P. psidii	HW-2	*S. paniculatum*	Urediniospores	Hawaii
P. psidii	BRIP 57997	*Eugenia reinwardtiana*	Urediniospores	Australia
P. psidii	BRIP 57991	*Melaleuca leucadendra*	Urediniospores	Australia
P. psidii	BRIP 57985	*S. jambos*	Urediniospores	Australia
P. psidii	BRIP 57793	*Rhodamnia angustifolia*	Urediniospores	Australia
P. psidii	BRIP 57922	*M. quinquenervia*	Urediniospores	Australia
P. psidii	BRIP 58000	*Rhodamnia rubescens*	Urediniospores	Australia
P. psidii	BRIP 58037	*Backhousia citriodora*	Urediniospores	Australia
P. psidii	BRIP 58038	*M. viridiflora*	Urediniospores	Australia
P. psidii	BRIP 58511	*Rhodomyrtus psidioides*	Infected tissue	Australia
P. psidii	BRIP 58513	*Rhodamnia rubescens*	Infected tissue	Australia
P. psidii	T13_03489A	*S. jambos*	Infected tissue[a]	New Caledonia
P. psidii	T13_03489B	*E. gacognei*	Infected tissue[a]	New Caledonia
P. graminis	PDD101636	*Festuca arundinacea*	Urediniospores	New Zealand
P. striiformis	PDD101676	*Dactylis glomerata*	Urediniospores	New Zealand
P. hordi	MP273	*Holcus lanatus*	Urediniospores	New Zealand
P. hemerocallidis	PDD102320	*Hemerocallis* sp.	Urediniospores	New Zealand
P. oxalidis	Unknown	*Oxalis* sp.	Urediniospores	New Zealand
P. coronata	T12_03234	*Lolium* sp.	Urediniospores	New Zealand
P. myrsiphylli	T10_03433A	*Asparagus asparagoides*	Urediniospores	New Zealand

[a] Herbarium specimens obtained from New Caledonia.

ITS and β-tubulin consensus sequences using Beacon Designer 8.01 software (Premier Biosoft) with default settings for standard TaqMan design. The primers and probes were evaluated in silico for potential secondary structures using a Beacon Designer in order to select suitable forward and reverse primer combinations. Primer pairs were selected to amplify a very short PCR fragment (<200 bp), because they amplify more efficiently and are more tolerant of reaction conditions. The hydrolysis probes were designed with 6-carboxy-fluorescein (FAM) fluorophore at the 5′ end and Black Hole Quencher-1 (BHQ-1) at the 3′ end. The specificity of the ITS and β-tubulin primers and probes was evaluated by in silico analysis using a BLASTn search. Only primers and probes with high specificity to *P. psidii* were synthesized by BioSearch Technologies and used in this study (Table 3).

Development and optimization of qPCR assays. The *P. psidii* qPCR assays were performed using a LightCycler 480 Probes Master PCR Mix (Roche). All the qPCR assays were carried out in a final volume of 20 μl: 10 μl of 2× reaction buffer, a final concentration of 300 nM each specific forward and reverse primer, 120 nM FAM- or BHQ-labeled TaqMan probe (Table 3), bovine serum albumen at 0.5 μg/μl, $MgCl_2$ (5 mM final), and 2 μl of DNA template. Thermocycling conditions were as follows: 95°C for 3 min, then 40 cycles of 95°C for 15 s and 60°C for 45 s. All three qPCR assays were further optimized with the addition of extra $MgCl_2$ (4.2 to 6.2 mM final concentration) and gradient qPCR (55 to 65°C) to obtain an optimum annealing temperature for each assay. The PCR amplification quality of the DNA extracted from healthy and *P. psidii*-infected plant tissues was checked by using plant internal control primers and probes that target the cytochrome oxidase (*cox*) gene (Weller et al. 2000) using the above PCR reagents and conditions.

Specificity and analytical sensitivity of qPCR assays. Specificity of the three qPCR assays were tested against all *Puccinia* isolates in Table 1 and against 13 plant species in the Myrtaceae family commonly found in New Zealand (Table 2). The sensitivity of all three qPCR assays was evaluated using 10-fold serial dilutions of *P. psidii* genomic DNA from known concentrations prepared in sterile water.

Table 2. Healthy Myrtaceae plants species tested for specificity of real-time polymerase chain reaction assays

Common name	Scientific name
Acmena	*Syzygium smithii*
Agonis	*Agonis fleuosa*
Bottle bush	*Callistemon* sp.
Eucalyptus	*Eucalyptus* sp.
Eugina	*Syzygium smithii*
Fijoa	*Acca sellowlana*
Guava	*Psidium cattleianum*
Kanuka	*Kunzea ericoides*
Manuka	*Leptospermum scoparium*
Melaleuca	*Melaleuca* sp.
Pohutukawa	*Metrosideros excelsa*
Ramarama	*Lophomyrtus bullata*
Rata	*Metrosideros carminea*

The detection sensitivity of the qPCR assays was also tested against *P. psidii* genomic DNA (0.5 ng/μl) spiked into healthy *Eucalyptus* sp. DNA (35 ng/μl) at different ratios from 1:1 to 1:20 (*P. psidii*/*Eucalyptus* sp.). The sensitivities of the qPCR assays were then further compared against an existing conventional nested PCR detection tool for *P. psidii* using the method developed by Langrell et al. (2008). The same 10-fold serial dilutions of *P. psidii* genomic DNA of known concentrations prepared in sterile water were used as templates for a nested PCR assay for comparison.

In planta detection of *P. psidii* was assessed by selecting the qPCR assay with the greatest detection sensitivity to test rust-infected plant materials collected in Australia and New Caledonia. The qPCR assay PpsiITS1 (with primer pair PpsiITS1F/PpsiITS1R and TaqMan probe PpsiITS1P) was used to detect *P. psidii* from 40 DNA extractions from myrtle-rust-infected plants of *Rhodomyrtus psidioides and Rhodamnia rubescens* from Australia and four DNA extractions from myrtle-rust-infected herbarium material of *S. jambos* and *E. gocognei* from New Caledonia. These DNA extracts from rust-infected plant tissues were also tested with the conventional nested PCR assay (Langrell et al. 2008).

Assay repeatability and reproducibility. Assay repeatability and reproducibility were determined for the qPCR assay PpsiITS1 by calculating percent coefficient of variation (%CV) for intra- and inter-assay using the mean cycle threshold (Ct) values and standard deviations. To assess repeatability, the intraassay CV was calculated from eight replicates of three dilutions (500, 5, and 0.5 pg/μl) of *P. psidii* DNA extracted from urediniospores tested in the same run.

The assay reproducibility was assessed by calculating interassay CV from three consecutive qPCR runs performed on different days by three different operators. To demonstrate transfer of the assay to other laboratories, reproducibility was assessed by using different reagents and real-time PCR machines in different laboratories in New Zealand.

Development of duplex assay. A duplex qPCR assay was then developed to coamplify *P. psidii* DNA with the plant *cox* gene as an internal control. This duplex qPCR was developed using primers PpsiITS1F and PpsiITS1R and probe PpsiITS1P (Table 3) and the *cox* gene-specific primers and probe (Weller et al. 2000). The duplex qPCR was performed using a PerfeCTa qPCR ToughMix (Quanta Biosciences) and with an additional PCR reagent "COX BLOCK" (dNature Diagnostics & Research Ltd.). The COX BLOCK is specific to the internal control (*cox* gene) amplicon and is used to delay the amplification of the *cox* gene, thereby preventing the internal control from outcompeting the detection of *P. psidii* DNA. The duplex qPCR assay was carried out in a final volume of 20 μl: 10 μl of 2× ToughMix, a final concentration of 300 nM each specific forward and reverse primer for *P. psidii* and the *cox* gene, 120 nM FAM-labeled TaqMan probe for *P. psidii* and 100 nM CAL Fluor Red 610-labeled TaqMan probe for the *cox* gene, with 0, 300, 500, and 750 nM concentrations of COX BLOCK and 2 μl of DNA template. The thermocycling conditions used were as follows: 95°C for 3 min, then 40 cycles of 95°C for 5 s and 59°C for 30 s. Tenfold serial dilutions of *P. psidii* DNA spiked with healthy *Eucalyptus* sp. DNA as diluents were used as template for duplex qPCR assay to determine an optimum COX BLOCK concentration at which the

Table 3. *Puccinia psidii*-specific primers and TaqMan probes used in this study

Assay name	Primer, probe[a]	Sequence (5′–3′)	Target
PpsiITS1	PpsiITS1F	GTAGCTTTATTGAAACATAGTAA	ITS1
	PpsiITS1R	TGATTTTAGACAATAATAATAAGGG	…
	PpsiITS1P	FAM-AGATTAATATCTTTGCCACGTATACCA-BHQ1	…
PpsiITS2	PpsiITS2F	ACCACTTCTACCTTATTACA	ITS1
	PpsiITS2R	GGGTAATGTGACAAAGAT	…
	PpsiITS2P	FAM-TGCCACGTATACCAAATTACTATGTTT-BHQ1	…
PpsiBT1	PpsiBT1F	AGGTCAACTCAACTCTGA	β-Tubulin
	PpsiBT1R	GAGGTTAGCTCTGGTACA	…
	PpsiBT1P	FAM-ACGATATTGCTGGCTACCACGA-BHQ1	…

[a] F = forward primer, R = reverse primer, and P = TaqMan probe.

target Ct values are comparable in both single and duplex qPCR assays. The Ct values obtained for the target (*P. psidii*) and *cox* gene amplifications were compared between single and duplex qPCR assays. The optimized duplex qPCR assay for *P. psidii* and the *cox* gene was then tested using DNA extracted from naturally myrtle-rust-infected plant tissues.

Results

***P. psidii*-specific primers and probes.** Three new sets of qPCR primers and probes, including two from the ITS region and one from the β-tubulin gene, were designed for *P. psidii* in this study. The amplified ITS1 and the β-tubulin regions were found to be highly conserved within the *P. psidii* population. The in silico BLASTn analysis confirmed that the newly designed primer and probe sequences were specific (100% homology) to the target species *P. psidii* only.

Development of *P. psidii* qPCR assays. The designed qPCR primers and probes with LightCycler 480 Probes Master Mix specifically amplified the *P. psidii* target sequence only. For qPCR assays PpsiITS1 and PpsiITS2, optimum amplification was achieved by the addition of $MgCl_2$ (4.2 mM final concentration). However, additional $MgCl_2$ did not change the amplification performance of the β-tubulin qPCR assay. Optimum annealing temperatures were identified in gradient qPCR assays for PpsiITS1 and PpsiITS2 qPCR assays at 59°C and for the PpsiBT1 assay at 60°C. All three qPCR-amplified products were analyzed in gel electrophoresis and this confirmed that all amplifications resulted in a single PCR product, as expected (data not shown). The sequence analysis of the amplified PCR products of these three qPCR assays showed that all amplicons were 100% identical to *P. psidii* sequences in GenBank (AJ421800 and EU487246).

Specificity of *P. psidii* qPCR assays. Specificity tests of qPCR assays PpsiITS1, PpsiITS2, and PpsiBT1 against 13 *P. psidii* isolates from different countries confirmed that these assays were able to detect all *P. psidii* strains regardless of their geographical origin. No cross amplifications were produced in any of the other seven *Puccinia* spp. tested in this study. Specificity tests of the qPCR assays PpsiITS1, PpsiITS2, and PpsiBT1 against 13 plant species in the Myrtaceae family present in New Zealand did not cross amplify.

Sensitivity of qPCR assays. Analytical sensitivities for qPCR assays PpsiITS1 and PpsiITS2 were 0.011 and 1.1 pg of *P. psidii* genomic DNA, respectively. This result showed that the qPCR assay PpsiITS1 was at least 100 times more sensitive than the PpsiITS2 assay. The analytical sensitivity obtained for qPCR assay PpsiBT1 was 110 pg of *P. psidii* DNA (Fig. 2). The conventional nested PCR assay detected down to 1.1 pg of *P. psidii* DNA (Fig. 3). The sensitivity obtained for the conventional nested PCR was equal to the sensitivity of the qPCR assay PpsiITS2 and was 100-fold less sensitive than the qPCR assay PpsiITS1.

All three qPCR assays were able to detect *P. psidii* in the presence of host (*Eucalyptus* sp.) DNA down to a 1:20 dilution (Table 4). These results show that all three qPCR assays were sensitive enough to detect *P. psidii* in host tissues. Similar to previous results, the Ct values for the PpsiBT1 qPCR assay were higher than the Ct values obtained for PpsiITS1 and PpsiITS2 assays (Table 4).

The best-performing qPCR assay PpsiITS1 was able to detect *P. psidii* from all the rust-infected *Rhodomyrtus psidioides* and *Rhodamnia rubescens* samples collected from Australia and herbarium samples of *S. jambos* and *E. gacognei* from New Caledonia (Table 5). The qPCR assay PpsiITS1 was also able to detect *P. psidii* from nonsymptomatic *Rhodomyrtus psidioides* leaves collected from an infected tree. The conventional *P. psidii* nested PCR assay also detected *P. psidii* in all rust-infected samples (Table 5).

Assay repeatability and reproducibility. The repeatability experiments for the qPCR assay PpsiITS1 across eight replicates of three dilutions (500, 5, and 0.5 pg/μl) of *P. psidii* DNA confirmed that the assay was repeatable and reproducible. The intra- and inter-assay CV for the qPCR assay PpsiITS1 were significantly low (0.7 to 1.3 and 0.8 to 1.6, respectively; Table 6). Reproducibility of this qPCR assay was further confirmed as the assay was tested by using three different operators on different days in our laboratory and independently in different laboratories in New Zealand. Reproducible results were consistently obtained for the qPCR assay PpsiITS1 tested using the DNA extracted from urediniospores and *P. psidii*-infected samples from different laboratories in New Zealand (Table 7).

Development of duplex qPCR assay. The duplex assay developed for *P. psidii* and the *cox* gene was able to produce amplification of both targets in serial-diluted *P. psidii* DNA prepared in *Eucalyptus* sp. DNA. The Ct values obtained for *P. psidii* did increase when the duplex assay was tested at lower concentrations of *P. psidii* DNA with the addition of COX BLOCK at 0, 300, and 500 nM

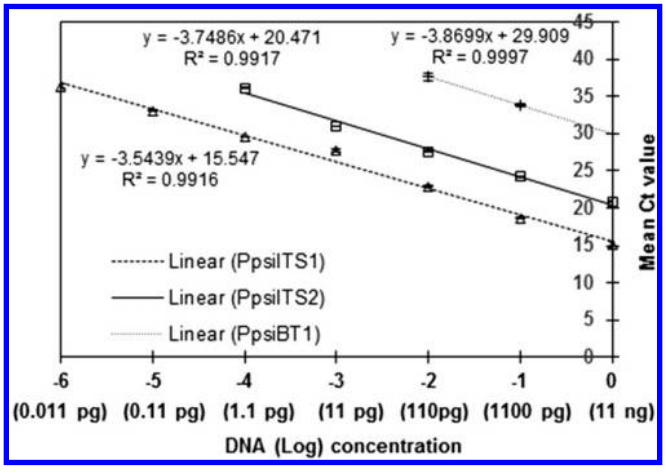

Fig. 2. Sensitivities of the *Puccinia psidii* real-time polymerase chain reaction assays (PpsiITs1, PpsiITS2, and PpsiBT1) for detecting *P. psidii* using seven 10-fold serial dilutions ($n = 3$) of purified DNA. Coefficients of determination R^2 and the slopes of each regression curve are indicated; Ct = cycle threshold.

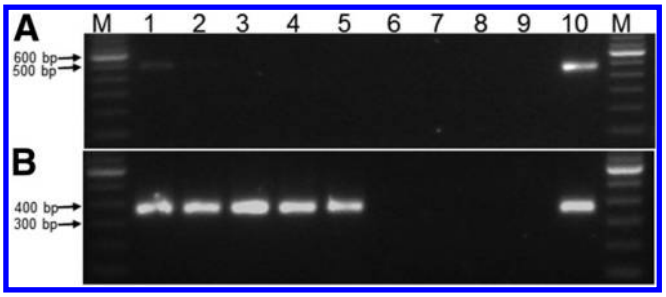

Fig. 3. Sensitivity of the *Puccinia psidii* nested polymerase chain reaction (PCR) assay. **A,** First PCR amplification with primers Ppsi1 and Ppsi6 and **B,** nested PCR amplification with primers Ppsi2 and Ppsi4. Lane 1, 11 ng DNA; lane 2, 1.1 ng DNA; lane 3, 110 pg DNA; lane 4, 11 pg DNA; lane 5, 1.1 pg DNA; lane 6,110 fg DNA; lane 7, 11 fg DNA; lane 8, 1.1 fg DNA; lane 9, negative control; lane 10, *P. psidii* positive control; and lane M, 100-bp marker (Invitrogen).

Table 4. Polymerase chain reaction (PCR) detection of *Puccinia psidii* using real-time PCR assays PpsiITS1, PpsiITS2, and PpsiBT1from host (*Eucalyptus* sp.) DNA spiked with *P. psidii* DNA at different ratios

	Ct values for each qPCR[a]			
DNA template mixture[b]	PpsiITS1	PpsiITS2	PpsiBT1	*cox* Gene
1:0	20.76	23.08	30.67	NA
1:1	22.08	24.50	31.64	22.88
1:5	23.65	26.15	33.24	22.69
1:10	24.59	27.03	34.19	22.66
1:15	25.14	27.46	34.72	22.75
1:20	25.38	27.70	35.20	23.14
0:1	NA	NA	NA	23.06
Water	NA	NA	NA	NA

[a] NA = not amplified.
[b] Ratio of *P. psidii* (0.5 ng/μl) to *Eucalyptus* sp. (35 ng/μl).

(Table 8). However, the Ct values obtained for *P. psidii* in duplex assays with 750 nM and 1 μM COX BLOCK were similar to those in a *P. psidii* single qPCR assay (Table 8). The COX BLOCK addition to the duplex reactions at 500 and 750 nM significantly delayed the *cox* gene amplification but 1 μM COX BLOCK almost blocked the amplification. Thus, the duplex qPCR protocol for *P. psidii* and the *cox* gene was optimized with the addition of 750 nM COX BLOCK. The optimized duplex qPCR assay was able to efficiently detect *P. psidii* from all myrtle-rust-infected plant tissues tested in this study.

Discussion

The aim of this study was to develop a highly sensitive, species-specific qPCR method that would allow for high-throughput detection of *P. psidii* directly from infected plant tissues. Novel TaqMan qPCR primers and probes based on ITS and β-tubulin DNA sequences have been developed and validated for the detection of *P. psidii*. All three qPCR assays were able to specifically detect *P. psidii* DNA extracted from urediniospores as well as from infected plant tissues.

The ITS region was used as our primary target to design *P. psidii*-specific qPCR primers and probes because the ITS has been reported to provide a genomic region that is variable enough to differentiate between rust species (Maier et al. in press) and has been shown to be useful to develop specific PCR primers to detect *P. psidii* (Langrell et al. 2008). In addition, we also developed specific primers and probes that target β-tubulin sequences because they have been shown to be of value for species-specific qPCR for several fungal species (Aroca et al. 2008). The specificity of all three qPCR assays was extensively tested and shown to specifically amplify *P. psidii* from infected Myrtaceae plants. All three qPCR assays were able to detect *P. psidii* from all voucher samples regardless of their host and geographical origin. This included strains collected from different Myrtaceae plant species from Australia, Brazil, Hawaii, and New Caledonia. No cross amplification was produced from testing healthy

Table 5. Detection of *Puccinia psidii* from infected Myrtaceae plant samples with real-time polymerase chain reaction (PCR) assay (PpsiITS1) and nested PCR

Sample	Host	Origin	Infection level[a]	PpsiITS1 qPCR (Ct)	*cox* Gene qPCR (Ct)[b]	Nested PCR[c]
1	*Rhodomyrtus psidioides*	Australia	A	33.75	25.96	+
2	*R. psidioides*	Australia	A	31.95	23.80	+
3	*R. psidioides*	Australia	A	31.46	24.30	+
4	*R. psidioides*	Australia	B	28.55	27.91	+
5	*R. psidioides*	Australia	B	29.21	27.17	+
6	*R. psidioides*	Australia	B	29.01	26.76	+
7	*R. psidioides*	Australia	C	28.71	26.74	+
8	*R. psidioides*	Australia	C	30.56	26.12	+
9	*R. psidioides*	Australia	C	29.94	25.91	+
10	*R. psidioides*	Australia	D	19.61	24.76	+
11	*R. psidioides*	Australia	D	23.38	26.40	+
12	*R. psidioides*	Australia	D	27.57	27.63	+
13	*R. psidioides*	Australia	A	33.87	21.06	+
14	*R. psidioides*	Australia	A	34.98	22.60	+
15	*R. psidioides*	Australia	A	32.06	22.22	+
16	*R. psidioides*	Australia	B	28.39	28.90	+
17	*R. psidioides*	Australia	B	29.76	26.03	+
18	*R. psidioides*	Australia	B	26.12	27.63	+
19	*R. psidioides*	Australia	C	26.24	27.16	+
20	*R. psidioides*	Australia	C	31.08	28.82	+
21	*R. psidioides*	Australia	C	30.80	26.12	+
22	*R. psidioides*	Australia	D	17.59	21.94	+
23	*R. psidioides*	Australia	D	31.32	27.45	+
24	*R. psidioides*	Australia	D	22.88	25.14	+
25	*R. psidioides*	Australia	D	20.78	22.18	+
26	*R. psidioides*	Australia	D	20.45	22.33	+
27	*R. psidioides*	Australia	D	28.69	23.75	+
28	*R. psidioides*	Australia	D	27.30	27.20	+
29	*R. psidioides*	Australia	D	22.54	21.86	+
30	*R. psidioides*	Australia	D	18.14	22.10	+
31	*R. psidioides*	Australia	D	28.34	20.47	+
32	*R. psidioides*	Australia	D	21.86	21.37	+
33	*Rhodamnia rubescens*	Australia	A	35.03	22.36	+
34	*R. rubescens*	Australia	A	33.42	23.72	+
35	*R. rubescens*	Australia	B	23.12	21.52	+
36	*R. rubescens*	Australia	B	23.15	22.90	+
37	*R. rubescens*	Australia	C	22.27	23.81	+
38	*R. rubescens*	Australia	C	19.04	22.20	+
39	*R. rubescens*	Australia	D	19.37	22.84	+
40	*R. rubescens*	Australia	D	27.97	24.42	+
41	*Syzygium jambos*	New Caledonia	D	17.76	24.40	+
42	*S. jambos*	New Caledonia	D	17.72	23.98	+
43	*Eugenia gocognei*	New Caledonia	D	19.24	24.74	+
44	*E. gocognei*	New Caledonia	D	18.29	23.54	+
45	Sterile water	…	…	…	…	−

[a] Infection levels are visually estimated percentages of leaf area covered with rust pustules. A = asymptomatic (0%), B = <15% leaf area covered with rust pustules, C = 15 to 30% leaf area covered with rust pustules, and D = >30% leaf area covered with rust pustules.
[b] qPCR amplification of plant internal control (*cox* gene) to check the quality of DNA extractions.
[c] Species-specific PCR assay (Langrell et al. 2008): + = presence of the specific PCR amplicon and − = absence of the specific PCR amplicon.

Myrtaceae plants or other closely related *Puccinia* spp. Recent studies using microsatellite markers to investigate the genetic diversity within *P. psidii* reported the occurrence of different genotypes in Brazil and a single genotype in Hawaii (Graça et al. 2011; Zhong et al. 2011). We are confident that our developed qPCR assays detect all strains because we have tested a number of *P. psidii s*amples from different countries and different hosts. Furthermore, Langrell et al. (2008) found high levels of homogeneity in sequences of the ITS region among *P. psidii* regardless of host or geographic origin or genotype designation.

The detection sensitivities of the qPCR assays developed in this study varied significantly, where both ITS qPCR assays were 100 times more sensitive than the β-tubulin qPCR. This was expected because rDNA regions provide excellent detection targets due to their occurrence in multiple copies throughout the fungal genome, thereby enabling highly sensitive PCR amplification. It has been estimated that approximately 45 to 150 copies of the ITS region can be present in one haploid fungal genome and, for most species, it is around 50 copies (Ganley and Kobayashi 2007). The qPCR assay PpsiITS1 showed greater analytical sensitivity than the other two qPCR assays and was selected as the best-performing qPCR assay. Although the detection sensitivity of the β-tubulin qPCR assay was low, this assay will still be useful as a second gene confirmatory assay for new detections of *P. psidii*. The detection level of nested PCR assay for *P. psidii* was reported to be approximately 0.1 pg (Langrell et al. 2008), which is theoretically equal to one urediniospore of *P. graminis* f. sp. *tritici* (Backlund and Szabo 1993). In this study, sensitivity obtained for the previously published *P. psidii*-specific nested PCR assay was 1.1 pg of genomic DNA. The difference in reported detection limits could be due to several factors, including different diseased plant tissue types, DNA extraction methods, or PCR reagents, or variation between PCR instruments. However, the sensitivity of the *P. psidii* qPCR assay PpsiITS1 was 10 times more sensitive when compared with the previously published nested PCR assay for *P. psidii*.

The qPCR assays enabled specific and sensitive detection of *P. psidii* in the presence of host DNA. Initially, the qPCR assays were evaluated using DNA from host plant tissue artificially spiked with *P. psidii* DNA due to unavailability of naturally infected plants in New Zealand. Later, the best-performing qPCR assay PpsiITS1 was tested in Australia and New Zealand on *P. psidii*-infected plant material and herbarium samples of myrtle-rust-infected plant material collected from a recent outbreak in New Caledonia. All these samples were from different Myrtaceae species and the qPCR assay PpsiITS1 was able to consistently detect *P. psidii* from all samples, including nonsymptomatic leaf tissue collected from an infected plant. The repeatability and reproducibility of this assay was further confirmed in our laboratory and by testing in two other laboratories in New Zealand and Australia (data not shown), and the results were comparable. In general, the assays showed a high level of robustness because using different thermocyclers or changing DNA extraction method and PCR reagents had little influence on overall results.

In addition, the qPCR assays described here incorporate the use of an internal control to ensure that the quality of the nucleic acid extracted from plant tissue is PCR competent, thereby eliminating false-negative results due to inhibition of the PCR. A plant internal PCR control based on primers to amplify the *cox* gene has been commonly used to check the PCR quality of nucleic acid extractions (Weller et al. 2000). In most cases, two separate PCR amplifications (target and *cox* gene) are required, which necessitates additional reagents and time. In this study, a duplex qPCR assay developed for *P. psidii* and the *cox* gene using an additional PCR ingredient (COX BLOCK) was able to amplify both targets in a single reaction without compromising the analytical sensitivity of the *P. psidii* single qPCR assay. The COX BLOCK is a patented PCR ingredient (dNature Diagnostics & Research, Ltd.) which delays the *cox* gene amplification until a substantial amount of target (pathogen) amplification is achieved. The duplex assay was tested against the nucleic acid extracted from myrtle-rust-infected plant materials and the results were comparable with *P. psidii* single qPCR detection. Compared with the previously published conventional nested PCR assay for *P. psidii*, both the single and duplex qPCR assays are faster and are less prone to contamination because no postamplification processing steps are required.

Table 6. Intra- and interassay coefficients of variation (CV) based on the mean cycle threshold values calculated for the real-time polymerase chain reaction assay PpsiITS1 tested with eight replicates of each *Puccinia psidii* DNA

	CV (%)	
***P. psidii* DNA (pg/µl)**	**Intraassay**	**Interassay**
500	1.3	1.3
5	0.7	0.8
0.05	1.1	1.6

Table 7. Interlaboratory comparison of Cycle threshold (Ct) values for the real-time polymerase chain reaction assay PpsiITS1 tested in different laboratories using different *Puccinia psidii* DNA extractions[a]

			DNA (10^{-1})		DNA (10^{-2})	
Sample	**Host**	**DNA from**	**Lab 1**	**Lab 2**	**Lab 1**	**Lab 2**
1	*Melaleuca viridiflora*	Urediniospores	23.20	22.66	26.10	26.06
2	*Rhodomyrtus psidioides*	Infected tissue	27.57	26.29	31.37	31.00
3	*Syzygium jambos*	Infected tissue	24.11	23.24	27.74	27.11
4	*S. jambos*	Infected tissue	24.48	24.16	27.37	28.39

[a] Lab 1 performed at Plant Health and Environment Laboratory (PHEL), New Zealand laboratory using LightCycler 480 Probes master mix (Roche) in CFX96 Touch Real-Time RCR Detection System(Bio-Rad) and Lab 2 performed at Scion, New Zealand using KAPA PROBE FAST qPCR kit (Custom Science) in Illumina Eco Real-Time PCR System.

Table 8. Comparison of cycle threshold (Ct) values for *Puccinia psidii* and the *cox* gene for real-time polymerase chain reaction (qPCR) assay PpsiITS1 and duplex qPCR assay with different concentrations of COX BLOCK

		Concentrations of COX BLOCK in duplex qPCR[a]							
	qPCR	**0**		**500 nM**		**750 nM**		**1 µM**	
DNA (pg/µl)[b]	***Ppsi***	***Ppsi***	***cox***	***Ppsi***	***cox***	***Ppsi***	***cox***	***Ppsi***	***cox***
500	20.37	21.22	18.75	20.25	22.38	21.96	31.60	20.34	32.65
50	23.83	26.71	18.67	24.12	22.26	24.31	29.15	23.30	31.11
5	27.29	30.76	18.34	29.16	22.24	27.37	28.77	27.54	31.06
0.5	30.87	34.62	18.37	34.02	22.27	30.72	28.58	30.49	31.06

[a] Ct values of *P. psidii* amplification (*Ppsi*) and plant *cox* gene amplification (*cox*).
[b] *P. psidii* DNA concentration, consisting of *P. psidii* genomic DNA diluted in a background of *Eucalyptus* sp. DNA.

The duplex qPCR assay for *P. psidii* had a shorter run time (55 min) than the *P. psidii* single qPCR assay (1 h and 15 min) and the conventional nested-PCR assay (6 h). This duplex qPCR assay will be particularly useful to facilitate high-throughput detection by increasing the number of samples that could be tested in a single qPCR run with the potential to process and analyze up to 96 samples within 4 h.

P. psidii is a quarantine pathogen in many countries, including New Zealand, and it is important to have fast and accurate detection tools to identify this pathogen in order to maximize the chances of a successful containment or eradication. Detection of a quarantine pathogen during an outbreak is time sensitive and may require hundreds of samples to be immediately tested. In previous responses, we have found that using conventional PCR is particularly cumbersome for high-throughput detection; for example, detection of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit (Taylor et al. 2014). Some of the main disadvantages of using conventional PCR were a reduced detection sensitivity compared with qPCR, increased risk of cross contamination or false positive results from PCR product carryover, and the use of electrophoresis in the detection phase resulting in longer processing times. During the *P. syringae* pv. *actinidiae* response, there was only a conventional PCR assay available for the detection of *P. syringae* pv. *actinidiae*. Significant resources were required to rapidly develop and validate a qPCR assay to test thousands of kiwifruit samples within a short period of time. This removed resources from other response activities and, ultimately, delayed test results, emphasizing the need for continual improvement of diagnostic protocols for the high-throughput detection of quarantine pathogens.

Early and sensitive detection of myrtle rust will provide one of the few opportunities to prevent establishment of this disease. We believe that qPCR could provide a method of detection to identify infected material prior to fungal sporulation and, thereby, reduce potential spread of the fungus. New Zealand is currently free of myrtle rust; however, since the first detection of *Puccinia psidii* in Australia, the country is under threat of an incursion from this pathogen. A study by Kriticos and Leriche (2008) indicated that much of the North Island and some of the South Island of New Zealand appear to be climatically suitable for *P. psidii*. Similar to other *Puccinia* spp., *P. psidii* can spread by wind dissemination of infectious urediniospores from infected areas. Detection by microscopic examination of urediniospores collected from spore traps has been used in the field to estimate the wind dispersal of *P. psidii* urediniospores and to monitor the spread of myrtle rust disease in Brazil (Zauza et al. 2015). The *P. psidii* qPCR assays developed in this study could be used along with spore traps for more accurate and sensitive detection of *P. psidii* in monitoring high-risk areas. Other studies using qPCR to detect and monitor rust fungi in sugarcane have successfully detected *Puccinia* spp. prior to uredinia development and symptom expression (Glynn et al. 2010). Such detections could prove useful for eradication, containment, or disease management strategies because it would allow the early destruction of infected material, or allow movement controls to be put in place and the use of protective fungicides to limit the spread of inoculum.

To our knowledge, this is the first qPCR assay developed for *P. psidii*, thereby providing a rapid and sensitive tool for its detection. This assay was demonstrated to be highly sensitive, specific, and reliable and was able to detect *P. psidii* before disease symptom expression and rust spore production. Consequently, qPCR will have the potential for use during presymptomatic monitoring of myrtle rust and for screening nursery stocks for import and exports. Presymptomatic screening of plant material in post entry quarantine or at high-risk surveillance sites may play an important role in successful containment or possible eradication of the myrtle rust pathogen.

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