PCR assays for the sugarcane rust pathogens *Puccinia kuehnii* and *P. melanocephala* and detection of a SNP associated with geographical distribution in *P. kuehnii*

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*Puccinia kuehnii* and *P. melanocephala* cause orange and brown rust of sugarcane, respectively. *Puccinia kuehnii* has been confirmed in Asia, Australia and recently, the Caribbean basin, whereas *P. melanocephala* is distributed among the majority of sugarcane growing regions. Differentiating these two economically significant pathogens visually is problematic and limited to material exhibiting mature disease symptoms or spores. Partial ITS1, ITS2 and complete 5.8S sequences were generated from *P. kuehnii* and *P. melanocephala* isolates from around the world. PCR primers and dual labelled hydrolysis probes were designed for each pathogen for use in real-time PCR and optimized using locked nucleic acids (LNA). The primers amplified DNA from their target pathogens and not from other species of *Puccinia* or fungal species isolated from sugarcane leaves. Optimized real-time PCR conditions allowed the detection of 0.19 pg of *P. kuehnii* or *P. melanocephala* genomic DNA and differentiated the pathogens on sugarcane leaves prior to observing typical symptoms in the field. Primer-introduced restriction analysis-PCR (PIRA-PCR) was used to detect a single nucleotide polymorphism (Pk ITS1 183A>G) in ITS1 of *P. kuehnii*. Allele 183A was observed in all samples, whereas 183G was detected in 52% of samples from Asia and Australia yet absent from all Caribbean basin samples. Long distance spore dispersal, dispersal through an intermediate location or improper movement of contaminated material could explain the introduction of *P. kuehnii* to the Western hemisphere. However, the current proliferation of the pathogen in the Americas is limited to isolates which contain only the 183A allele.

**Keywords:** brown rust of sugarcane, locked nucleic acids, orange rust of sugarcane, PIRA-PCR, real-time PCR, *Saccharum* spp.

**Introduction**

Two species of *Puccinia* can cause rust diseases of sugarcane (a complex hybrid of *Saccharum* spp.). *Puccinia kuehnii* is the causal agent of orange rust of sugarcane whereas *P. melanocephala* causes brown rust of sugarcane. Brown rust occurs in the majority of sugarcane growing regions: Africa, North and South America, South-East Asia, Southern Asia and Australasia (Ryan & Egan, 1989). Yield losses of 10–40% due to brown rust have been reported on susceptible varieties (Comstock & Shine, 1992). The first occurrence of *P. melanocephala* in the Western hemisphere was in the Dominican Republic in 1978 (Presley et al., 1978). Following this introduction, it spread rapidly through the sugarcane growing regions of North, Central and South America as well as the Caribbean and within a few years was present in almost all sugarcane-growing areas in the Americas (Purdy et al., 1985). Orange rust is less widespread than brown rust and was considered by Ryan & Egan (1989) as only occurring in the sugarcane industries in Asia and Australia. The recent report of orange rust in Florida was the first confirmed occurrence of the pathogen in the Western hemisphere (Comstock et al., 2008). The disease has since been confirmed in Guatemala (Ovalle et al., 2008), Nicaragua, Costa Rica (Chavarria et al., 2009), El Salvador, Panama and Mexico (Flores et al., 2009) and recently Brazil (Barbasso et al., 2010).

Economic losses caused by orange rust were estimated at AUST $150–200 M in an epidemic in Australia in

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2001 (Magarey et al., 2001). The spread of orange rust has so far been less rapid than that of brown rust. Currently with the exception of Brazil, South America and the sugarcane growing regions of the USA other than Florida remain free from orange rust. Additionally, there are no confirmed occurrences of orange rust in Africa. These areas are under considerable threat from this economically important pathogen. This makes the development of rapid and sensitive methods for detecting *P. kuehnii* critical in order to allow the rapid implementation of strategies that will limit the spread of the disease.

Since both *P. kuehnii* and *P. melanocephala* can occur together on sugarcane leaves and in the sugarcane growing environment, it is important to be able to accurately and reliably distinguish between the two pathogens. Differences in urediniospore coloration on uredinial lesions (pustules) are the most distinguishing morphological features between the two pathogens, with pustules of *P. kuehnii* typically light orange and those of *P. melanocephala* usually dark brown. Additionally, pustules caused by *P. kuehnii* are typically shorter and appear oval in shape whilst those caused by *P. melanocephala* are usually longer and narrower in appearance. These general differences in both urediniospore colour and shape of uredinia are often subtle and can vary depending upon age of infection, location and host cultivar. Microscopic examination of spore morphology can be used to identify urediniospores of *P. kuehnii* and differentiate them from those of *P. melanocephala* in well-preserved infected material that exhibit mature disease symptoms. However, these methods require a high power microscope and are less discriminatory when samples are preserved under sub-optimal conditions or when samples are taken during the initial stages of infection. Furthermore, these methods are difficult to apply to environmental samples, such as rain or soil, where the number of spores may be extremely low. Accurate identification of infection by sugarcane rust pathogens in the field is only possible when spores are produced from uredinial lesions.

Accurate identification can be achieved using molecular methods involving sequencing regions of rDNA. However, this can be time consuming and expensive to apply on a large scale. The development of quick, less expensive diagnostic methods are needed to allow for the accurate and sensitive discrimination between *P. kuehnii* and *P. melanocephala*. Regions of rDNA sequence have been widely exploited for the development of diagnostic methods for plant pathogens including several species of rust pathogens (Fraaije et al., 2001; Barnes & Szabo, 2007; Barnes et al., 2009). The advantage of using this region is that, due to the high number of tandem repeats of rDNA, detection methods targeting this region are highly sensitive.

The aims of this study were (i) to develop both conventional and real-time PCR assays for *P. kuehnii* and *P. melanocephala*, and (ii) to develop an assay that allows the detection of a single nucleotide polymorphism (SNP) identified in ITS1 of *P. kuehnii*.

**Materials and methods**

*Puccinia kuehnii* and *P. melanocephala* rDNA sequences

Sources of *P. kuehnii* and *P. melanocephala* were chosen to represent a geographically diverse population by incorporating most of the world sugarcane growing regions where these pathogens have been reported (Table 1). Sugarcane leaves exhibiting symptoms typical of orange rust caused by *P. kuehnii* or brown rust caused by *P. melanocephala* were removed from the plants, cut into sections approximately 15 cm in length, pressed flat between absorbent paper and placed in paper envelopes. After drying at room temperature for 1–2 days, samples were placed in paper envelopes and sent to the USDA, ARS, Systematic Mycology and Microbiology Laboratory, Beltsville, MD, USA.

DNA was isolated from 100 mg of dried leaf material containing uredinial lesions. Leaf material was chopped initially using a scalpel into pieces about 2 × 4 mm or smaller, placed in a tube containing lysing matrix ‘C’ (MP Biomedicals) and disrupted in a FastPrep (MP Biomedicals) using a speed setting of 4.5 for 30 s. Disruption was repeated up to four times until the leaf material took on the appearance of a fine powder. The remainder of the extraction procedure was performed using OmniPrep™ (G Biosciences) kits according to the manufacturer’s instructions. The resulting DNA pellets were re-suspended in the supplied TE buffer (G Biosciences). DNA concentration was determined by spectrophotometry and diluted to a final concentration of 40 ng µL⁻¹.

Primers PkPm-F and PkPm-R, located in ITS1 and ITS2, respectively, (Table 2) were designed from alignments of sequences accessioned in the National Center for Biotechnology Information (NCBI) GenBank database and are conserved between the two pathogens. These primers were used to amplify fragments of 606 bp (*P. kuehnii*) or 585 bp (*P. melanocephala*) from each DNA extract. PCR reactions were performed in volumes of 50 µL and contained 2·5 mM MgCl2, 0·25 mM dNTPs, 0·5 µM of each primer and 2·5 units *Taq* (Promega) and respective buffer. Thermocycling consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s and a final extension of 72°C for 7 min.

Amplified fragments were excised from agarose gels following electrophoresis, purified, and cloned. At least 10 positive clones were purified and sequenced for each sample. Sequences from each pathogen/location combination were edited and aligned using SEQUENCER version 4.7 (Genecodes).

**Species-specific primers**

Two sets of species-specific primers were designed. The first set was developed for use with standard thermocycling equipment and fragment visualization using agarose gel electrophoresis. The primers Pk1-F and Pk1-R...
Table 1 Sources and number of Puccinia kuehnii and P. melanocephala-infected sugarcane leaf samples used in this study and GenBank accession numbers for resulting rDNA sequences

<table>
<thead>
<tr>
<th>Country</th>
<th>Pathogen</th>
<th>No. samples</th>
<th>GenBank Acc. No.</th>
<th>Yeara</th>
<th>Cultivara</th>
<th>Locationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>P. kuehnii</td>
<td>2</td>
<td>GU564415, GU564419</td>
<td>2001</td>
<td>Q124</td>
<td>Mackay, QLD</td>
</tr>
<tr>
<td>China</td>
<td>P. kuehnii</td>
<td>2</td>
<td>GU564408, GU564417</td>
<td>2008</td>
<td>RB72–454</td>
<td>Guangxi Province</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>P. kuehnii</td>
<td>8</td>
<td>GU564409</td>
<td>2008</td>
<td>CP 72–2086</td>
<td>Guanacaste</td>
</tr>
<tr>
<td>Guatemala</td>
<td>P. kuehnii</td>
<td>2</td>
<td>GU564410</td>
<td>2007</td>
<td>BT 65–152</td>
<td>Santa Lucia, Cotzumalguapa</td>
</tr>
<tr>
<td>Jamaica</td>
<td>P. kuehnii</td>
<td>1</td>
<td>GU564411</td>
<td>2008</td>
<td>BT 9186</td>
<td>St. Catherine Parish, Middlesex County</td>
</tr>
<tr>
<td>Japan</td>
<td>P. kuehnii</td>
<td>8</td>
<td>GU564416, GU564420</td>
<td>2007</td>
<td>NCo310</td>
<td>Makabe, Okinawa</td>
</tr>
<tr>
<td>Mexico</td>
<td>P. kuehnii</td>
<td>3</td>
<td>GU564414</td>
<td>2008</td>
<td>NCo301</td>
<td>Chiapas</td>
</tr>
<tr>
<td>Nicaragua</td>
<td>P. kuehnii</td>
<td>3</td>
<td>GU564412</td>
<td>2007</td>
<td>ISA 00–1000</td>
<td>Ingieno San Antonio</td>
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<tr>
<td>Philippines</td>
<td>P. kuehnii</td>
<td>14</td>
<td>GU564413, GU564418</td>
<td>2007</td>
<td>56–226</td>
<td>Silay City, Negros Occidental</td>
</tr>
<tr>
<td>USA</td>
<td>P. kuehnii</td>
<td>6</td>
<td>GU564422</td>
<td>2009</td>
<td>CP 72–2086</td>
<td>Palm Beach County, Florida</td>
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<tr>
<td>Australia</td>
<td>P. melanocephala</td>
<td>2</td>
<td>GU564422</td>
<td>2007</td>
<td>Q117</td>
<td>Woodford, QLD</td>
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<td>Brazil</td>
<td>P. melanocephala</td>
<td>6</td>
<td>GU564423</td>
<td>2007</td>
<td>RB 92579</td>
<td>Pedro Alfonso, Tocantins</td>
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<tr>
<td>Colombia</td>
<td>P. melanocephala</td>
<td>13</td>
<td>GU564424</td>
<td>2008</td>
<td>MZC74–275 San Antonio de Los Caballeros, Cauca Valley</td>
<td></td>
</tr>
<tr>
<td>D.R. Congo</td>
<td>P. melanocephala</td>
<td>3</td>
<td>GU564434</td>
<td>2008</td>
<td>SP70–1284</td>
<td>200 km S. Kinshasa</td>
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<tr>
<td>Dominican Republic</td>
<td>P. melanocephala</td>
<td>4</td>
<td>GU564425</td>
<td>2008</td>
<td>CR9303</td>
<td>Higueral</td>
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<tr>
<td>Guadeloupe</td>
<td>P. melanocephala</td>
<td>4</td>
<td>GU564435</td>
<td>2008</td>
<td>B86517</td>
<td>Petit-bourg</td>
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<td>Guatemala</td>
<td>P. melanocephala</td>
<td>3</td>
<td>GU564426</td>
<td>2007</td>
<td>CG97–97</td>
<td>Santa Lucia, Cotzumalguapa</td>
</tr>
<tr>
<td>Jamaica</td>
<td>P. melanocephala</td>
<td>1</td>
<td>GU564431</td>
<td>2008</td>
<td>BT 9087</td>
<td>Scarlettie Piece</td>
</tr>
<tr>
<td>Mauritius</td>
<td>P. melanocephala</td>
<td>5</td>
<td>GU564330</td>
<td>2008</td>
<td>M 1202/01</td>
<td>Le Re'dult</td>
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<tr>
<td>Nicaragua</td>
<td>P. melanocephala</td>
<td>1</td>
<td>GU564427</td>
<td>2008</td>
<td>CP 72–2086</td>
<td>El Viejo, Puerto Morazán</td>
</tr>
<tr>
<td>Pakistan</td>
<td>P. melanocephala</td>
<td>4</td>
<td>GU564428</td>
<td>2007</td>
<td>SPF-234</td>
<td>Lalazar, Punjab</td>
</tr>
<tr>
<td>Panama</td>
<td>P. melanocephala</td>
<td>1</td>
<td>GU564432</td>
<td>2008</td>
<td>BT-34152</td>
<td>Natá, Coclé Province</td>
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<tr>
<td>Reunion</td>
<td>P. melanocephala</td>
<td>9</td>
<td>GU564433</td>
<td>2008</td>
<td>R 92/6261</td>
<td>St. Pierre</td>
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<tr>
<td>South Africa</td>
<td>P. melanocephala</td>
<td>6</td>
<td>GU564429</td>
<td>2007</td>
<td>N29</td>
<td>Mount Egdecombe, KwaZulu-Natal</td>
</tr>
<tr>
<td>USA</td>
<td>P. melanocephala</td>
<td>11</td>
<td>GU564436</td>
<td>2007</td>
<td>CP 05–1501</td>
<td>Palm Beach County, Florida</td>
</tr>
</tbody>
</table>

*aYear, cultivar and location within country given for samples with GenBank accession numbers.

Table 2 Targets for the primers and probes used in this study of Puccinia kuehnii and P. melanocephala and sizes of amplified products

<table>
<thead>
<tr>
<th>Assay Target</th>
<th>Name and sequencea</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probeb</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PkPm</td>
<td>P. kuehnii or P. melanocephala</td>
<td>PkPmF-aagagtgcacttaattgtggctc</td>
<td>PkPmR-tcccacctgatttgaggtct</td>
<td>N/A</td>
<td>606 or 585</td>
</tr>
<tr>
<td>Pk1</td>
<td>P. kuehnii</td>
<td>Pk1F-aagagtgcacttaattgtggctc</td>
<td>Pk1R-caggtaacaccttccttgatg</td>
<td>N/A</td>
<td>527</td>
</tr>
<tr>
<td>Pk2</td>
<td>P. kuehnii</td>
<td>Pk2F-gGgaacAcctcattattaacaagt</td>
<td>Pk2R-gcctagagacTattgga</td>
<td>N/A</td>
<td>142</td>
</tr>
<tr>
<td>Pk-PIRA</td>
<td>P. kuehnii</td>
<td>Pk-PIRAF-gcctagagacTattgga</td>
<td>N/A</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>Pm1</td>
<td>P. melanocephala</td>
<td>Pm1F-aattgtggctgaaaccttcetcc</td>
<td>Pm1R-ttgctactttccttgatg</td>
<td>N/A</td>
<td>480</td>
</tr>
<tr>
<td>Pm2</td>
<td>P. melanocephala</td>
<td>Pm2F-gtatacCaggtTaagtggc</td>
<td>Pm2R-gCctagagaTccttgga</td>
<td>N/A</td>
<td>130</td>
</tr>
</tbody>
</table>

bProbes were dual labelled with 6FAM (5’) and BHQ1 (3’).

Table 2 (generated a 527 bp product from P. kuehnii and Pm1-F and Pm1-R generated a 480 bp product from P. melanocephala DNA. Two additional forward primers were designed for use in real-time PCR assays, one specific to P. kuehnii (Pk2-F) and one to P. melanocephala (Pm2-F). The primers Pk2-F and Pk2-R (Table 2) generated a 142 bp product from P. kuehnii DNA and primers Pm2-F and Pm2-R (Table 2) generated a 130 bp product from P. melanocephala DNA.

The primers Pk1, Pk2, Pm1 and Pm2 were tested against DNA isolated from additional P. kuehnii and P. melanocephala infected leaves (Table 1) and DNA isolated from the rust causing pathogens P. coronata, P. graminis, P. recondita and Phakopsora pachyrhizi. Since secondary colonizing fungi are found on sugarcane leaves and among uredinia, the primers were also tested against fungi isolated from sugarcane leaves and from collections of uredinospores using the following procedure. Leaf pieces containing uredinial lesions were excised from P. kuehnii and P. melanocephala-infected
sugarcane leaves and plated onto potato dextrose agar (PDA). In addition, sugarcane leaves with orange or brown rust pustules were vacuumed to remove urediniospores together with any contaminating organisms present on the leaf surface or associated with the spores. The vacuumed material was retained in an Eppendorf tube, diluted in sterile distilled water (SDW) and plated onto PDA. Plates were incubated at room temperature for 7 days and the resulting colonies were plated onto fresh PDA plates, incubated for a further 7 days at room temperature and identified to genus level based on colony characteristics and morphology of fungal structures. DNA was extracted from the mycelium using the Omni-Prep™ (G Biosciences) kits described above and DNA was diluted to 20 ng μL⁻¹. The P. kuehnii and P. melanocephala primers were tested against DNA from these colonies using the above reaction conditions. DNA from each sample was tested using primers ITS4 and ITS5 (White et al., 1990) to confirm the quality of the extracted DNA.

Real-time PCR assays

Dual-labelled hydrolysis probes specific for each pathogen were designed for use with primers Pk2 and Pm2 for use in real-time assays. Probes consisted of the dye quencher BHQ1 attached at the 3’-end and the fluorescent label FAM at the 5’-end (Table 2). Primers Pk2 and Pm2 and the respective probes were designed manually from conserved positions within the sequence alignment of each pathogen and differing between P. kuehnii and P. melanocephala. Nucleotides with a 2'-O, 4'-C methylene bridge, known as locked nucleic acids (LNA) were used in the primers and probes. The number and positions of the LNA bases was optimized using EXIQON software available at http://lnatools.com/ with positions chosen to minimize self-hybridization and the generation of secondary structures. The primer and probe sequences including the positions of LNA bases are given in Table 2.

Real-time PCR reactions were performed using a Chromo4 real-time detection system (Bio-Rad) in 20 μL volumes containing 1× Premix Ex Taq™ (Perfect Real Time) (TaKaRa Bio. Inc.). Reaction conditions were optimized by performing gradients of annealing temperature (54–64°C), primer concentration (0.05–0.5 μM), and probe concentration (0.1–0.2 μM). The rDNA fragment amplified with primers PkPm-F/R and containing the species specific primer sites nested internally was excised from the gel following electrophoresis using Wizard® SV gel Clean-Up System (Promega). This was used as template DNA to optimize the real-time reaction conditions. The concentration of purified DNA was determined by spectrophotometry, diluted to an initial concentration of approximately 20 ng μL⁻¹ then along a serial dilution of 12 10-fold dilutions. Thermocycling conditions consisted of 98°C for 5 min followed by 45 cycles of 95°C for 30 s and the optimized annealing temperature for both primers of 60°C for 30 s followed by 72°C for 30 s. Reaction efficiencies for each condition were determined as a function of DNA concentration and change in cycle threshold (Ct) value calculated for each reaction (Gallup & Ackermann, 2006), efficiency slopes closest to -3.3 were selected as the optimum. Optimized concentrations of reaction ingredients were 0.5 μM (primers) and 0.1 and 0.2 μM, respectively, for the P. kuehnii and P. melanocephala probes. The sensitivity and detection range of the assays was then determined using P. kuehnii and P. melanocephala genomic DNA extracted from approximately 30 ng of urediniospores of each pathogen according to the procedures described for the leaf samples above. DNA was diluted to an initial concentration of 40 ng μL⁻¹ and then along a 10-fold serial dilution series.

Application of real-time PCR assays

Field inspections of sugarcane cultivars in the Canal Point breeding programme in South Florida were performed in June 2007 (when P. kuehnii was first observed in the region) and again in April 2009. The plots of several different cultivars showed a range of symptoms consistent with infection by sugarcane rust pathogens. Symptoms ranged from hypersensitive flecking to uredinial lesions on the underside of leaves. Where possible, the putative causal agent of the observed symptoms was recorded. Leaf parts from up to three separate leaves exhibiting these symptoms were excised from the leaves, dried and the DNA extracted from a 50 mg sample as described above. Further surveys for sugarcane rusts were performed in the same field plots several weeks following the taking of leaf samples. The susceptibility status of each cultivar towards both orange and brown rust was determined according to a rating scale originally described for brown rust by Tai et al. (1981) and recently adapted for both brown and orange rust (Comstock et al., 2010). A 0–4 rating scale was used where no pustules visible = 0 (highly resistant, HR), few poorly developed pustules = 1 (Resistant, R), more abundant pustules, larger in size = 2 (moderately resistant, MR), numerous large pustules spread over entire plant = 3 (moderately susceptible, MS), and numerous large-coalescing pustules, dead leaves = 4 (very susceptible, VS). DNA extracted from infected leaves was used in real-time PCR assays to detect P. kuehnii and P. melanocephala. Real-time PCR reactions were performed in triplicate using 100 ng of template genomic DNA and conditions as described above. Ct values for each reaction were recorded.

PIRA-PCR assay

A SNP in which nucleotide A is substituted for G was identified in ITS1 of P. kuehnii at position 183 of the 606 bp fragment generated using primers PkPm-F/R and is referred to as PkITS1 -183AsG. No restriction enzyme was available that recognizes this polymorphism, therefore a primer-introduced restriction analysis-PCR (PIRA-PCR) assay was developed. The primers Pk-PIRAF and Pk-PIRAR (Table 2) were designed using software described by Ke et al. (2001) and available at http://
cucumber leaves, vacuumed spore collections, or with host DNA isolated from sugarcane stalks (Table 3).

Reaction efficiencies for both assays and the dynamic range of amplification were similar using the purified rDNA or genomic DNA from *P. kuehnii* or *P. melanocephala* as a template (Fig. 1). Optimized reaction conditions for the *P. kuehnii* and *P. melanocephala* assays allowed amplification along a standard curve from 0.002 fg to 2000 fg using rDNA as a template and 0.00019 ng to 190 ng using genomic DNA as a template. The standard curves for both assays together with reaction efficiencies and slopes are given in Fig. 1.

**Application of real-time PCR assays**

The sugarcane leaf samples that exhibited disease symptoms identified as orange rust in 2007 yielded Ct values ranging from 17 to 21 using the *P. kuehnii* real-time assay (Table 4). Assays were negative using the *P. melanocephala* specific primer and probe, confirming *P. kuehnii* as the causal agent of disease. Orange rust pustules were subsequently observed on all cultivars sampled which were classified as resistant or moderately resistant to orange rust. Although no brown rust symptoms were

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**Results**

**Primer specificity and real-time assays**

Fragments of rDNA were successfully isolated from all the *P. kuehnii* and *P. melanocephala*-infected samples tested using primers PkPm-F and -R. The putative *P. kuehnii* and *P. melanocephala* specific primers designed from the alignments of these sequences only amplified DNA from each respective pathogen. Neither primer set cross-reacted with several other rust fungi tested or with fungal species isolated from infected sugarcane leaves, vacuumed spore collections, or with host DNA isolated from sugarcane stalks (Table 3).

Reaction efficiencies for both assays and the dynamic range of amplification were similar using the purified rDNA or genomic DNA from *P. kuehnii* or *P. melanocephala* as a template (Fig. 1). Optimized reaction conditions for the *P. kuehnii* and *P. melanocephala* assays allowed amplification along a standard curve from 0.002 fg to 2000 fg using rDNA as a template and 0.00019 ng to 190 ng using genomic DNA as a template. The standard curves for both assays together with reaction efficiencies and slopes are given in Fig. 1.

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**Table 3** Fungal species or genera used to test the *Puccinia kuehnii* and *P. melanocephala* specific primers developed in this study. The primers ITS4 and ITS5 (White et al., 1990) were used to confirm that the extracted DNA was amplifiable.

<table>
<thead>
<tr>
<th>Species or genus</th>
<th>Assay tested/result</th>
<th>P. kuehnii</th>
<th>P. melanocephala</th>
<th>ITS 4/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Botrytis sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cercospora sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cochliobolus sativus</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Darluca sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Epicoccum sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Penicilium sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Phakopsora pachyrhiza</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Puccinia coronata</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Puccinia graminis</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Puccinia striiformis</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pycicularia sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Rhizoctonia sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Zygomycota sp.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*DNA provided by the USDA-ARS Cereal Disease Laboratory.*

[Figure 1](#) Standard curves for (a) *Puccinia kuehnii* and (b) *P. melanocephala* real-time PCR assays using primers Pk2 and Pm2. Data points are DNA amount plotted against the mean cycle threshold (Ct).
observed on and no \( P. \) \( melanocephala \) DNA was detected in the leaves tested, brown rust pustules were observed on some of the cultivars later in the season and were classified as resistant or moderately resistant to brown rust (Table 4).

\( Puccinia \) \( kuehnii \) was detected in leaf samples from all six cultivars that exhibited flecking symptoms sampled in April 2009. Mean Ct values for these samples ranged from 22 to 36 (Table 4). Cultivars CP 07–2393 and CP 06–2664 were subsequently classified as highly resistant, CP 03–1491, CPCL 02–0908 and CPCL 06–3316 moderately resistant and CP 05–1463 moderately susceptible to orange rust based on visual symptoms. \( Puccinia \) \( kuehnii \) was also detected in two samples on which pustules were observed but could not be speciated visually, giving mean Ct values of 19 and 25 (Table 4). \( Puccinia \) \( melanocephala \) DNA was detected in leaf samples from all samples obtained from the Western hemisphere. Similarly, −183G was absent from all sequences generated in this study and in the NCBI database from the Western hemisphere. The detection frequencies of these two alleles in the sequences generated in this study together with results from the PIRA-PCR assay and in \( P. \) \( kuehnii \) sequences accessioned in the NCBI database, are given in Table 5. Allele −183A was observed in all 53 samples examined using the PIRA-PCR assay whereas −183G was observed only in 15 (52%) of the 29 samples from Australia, Japan, Philippines and China and absent from all samples obtained from the Western hemisphere. Similarly, −183G was absent from all sequences generated in this study and in the NCBI database from the Western hemisphere (Table 2). The detection frequencies of 183G:183A among samples from the Eastern hemisphere were 89% in the sequences generated in this study, 52% using the PIRA-PCR assay and 29% among sequences in the NCBI database.

**Discussion**

The first objective of this study was to develop conventional and real-time PCR assays for each of the two species of \( Puccinia \) which cause rust diseases of sugarcane. Sequencing isolates from almost all sugarcane growing regions combined with a strategy of sequencing multiple colonies from the cloning reactions increased the likelihood that the assays developed detect all isolates of the two pathogens. Testing the assays on DNA from fungal pathogens and saprophytes isolated from sugarcane leaves and spore collections also reduced the potential for cross reactions with other organisms associated with sugarcane.

<table>
<thead>
<tr>
<th>Year</th>
<th>Cultivar</th>
<th>Visual observations</th>
<th>( P. ) ( kuehnii ) (Ct)</th>
<th>( P. ) ( melanocephala ) (Ct)</th>
<th>Subsequent resistance rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>CPCL 02–2632</td>
<td>Orange rust</td>
<td>19.0 ± 0.32</td>
<td>−a</td>
<td>R (R1)b R (R1)</td>
</tr>
<tr>
<td>2007</td>
<td>CPCL 02–0320</td>
<td>Orange rust</td>
<td>17.4 ± 0.29</td>
<td>−</td>
<td>R (R1) HR (R0)</td>
</tr>
<tr>
<td>2007</td>
<td>CPCL 99–4295</td>
<td>Orange rust</td>
<td>18.0 ± 0.10</td>
<td>−</td>
<td>R (R1) R (R1)</td>
</tr>
<tr>
<td>2007</td>
<td>CP 03–1491</td>
<td>Orange rust</td>
<td>21.3 ± 0.40</td>
<td>−</td>
<td>MR (R2) MR (R2)</td>
</tr>
<tr>
<td>2007</td>
<td>CP 72–2086</td>
<td>Orange rust</td>
<td>21.4 ± 0.11</td>
<td>−</td>
<td>MR (R2) MR (R2)</td>
</tr>
<tr>
<td>2007</td>
<td>CP 89–2143</td>
<td>Orange rust</td>
<td>18.7 ± 0.41</td>
<td>−</td>
<td>MR (R2) R (R1)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 88–1762</td>
<td>Orange rust</td>
<td>17.7 ± 0.27</td>
<td>−</td>
<td>MS (R3) HR (R0)</td>
</tr>
<tr>
<td>2009</td>
<td>SP 89–1115</td>
<td>Orange rust</td>
<td>20.4 ± 0.06</td>
<td>−</td>
<td>MS (R3) MR (R2)</td>
</tr>
<tr>
<td>2009</td>
<td>N 29</td>
<td>Brown rust</td>
<td>−</td>
<td>25.7 ± 0.22</td>
<td>HR (R0) MS (R3)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 05–1658</td>
<td>Unidentified pustules</td>
<td>19.4 ± 0.38</td>
<td>−</td>
<td>MS (R3) HR (R0)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 05–1463</td>
<td>Flecking</td>
<td>21.8 ± 0.08</td>
<td>−</td>
<td>MS (R3) MR (R2)</td>
</tr>
<tr>
<td>2009</td>
<td>CPCL 05–6415</td>
<td>Unidentified pustules</td>
<td>25.3 ± 0.18</td>
<td>−</td>
<td>R (R1) R (R1)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 04–1367</td>
<td>Unidentified pustules</td>
<td>−</td>
<td>20.7 ± 0.14</td>
<td>HR (R0) R (R1)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 07–2393</td>
<td>Flecking</td>
<td>33.3 ± 0.74</td>
<td>−</td>
<td>HR (R0) HR (R0)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 03–1491</td>
<td>Flecking</td>
<td>36.0 ± 0.43</td>
<td>−</td>
<td>MR (R2) HR (R0)</td>
</tr>
<tr>
<td>2009</td>
<td>CPCL 02–0908</td>
<td>Flecking</td>
<td>35.2 ± 0.38</td>
<td>−</td>
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</tr>
<tr>
<td>2009</td>
<td>CPCL 06–3316</td>
<td>Flecking</td>
<td>34.7 ± 0.12</td>
<td>−</td>
<td>MR (R2) HR (R0)</td>
</tr>
<tr>
<td>2009</td>
<td>CP 06–2664</td>
<td>Flecking</td>
<td>35.9 ± 0.60</td>
<td>−</td>
<td>HR (R0) HR (R0)</td>
</tr>
</tbody>
</table>

a– = no Ct value observed.
bHS, highly susceptible; S, susceptible; MS, moderately susceptible; MR, moderately resistant; R, resistant; HR, highly resistant.

Number in brackets refers to 0–4 susceptibility rating scale.

---

The symptoms (Table 4).

*Real-time detection of \( Puccinia \) \( kuehnii \) and \( P. \) \( melanocephala \) using primers \( Pk2 \) and \( Pm2 \), respectively, in sugarcane leaves sampled in South Florida showing a range of symptoms.*

<table>
<thead>
<tr>
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<tr>
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<td>Flecking</td>
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<td>HR (R0) HR (R0)</td>
</tr>
</tbody>
</table>

\( ^a \) = no Ct value observed.

\( ^b \) HS, highly susceptible; S, susceptible; MS, moderately susceptible; MR, moderately resistant; R, resistant; HR, highly resistant.

Number in brackets refers to 0–4 susceptibility rating scale.

---

**SNP detection using PIRA-PCR**

The PIRA-PCR assay allowed the discrimination of the alleles of SNP \( PkITS1 \) −183A>G without the need for cloning and sequencing the respective fragments (Fig. 2). The frequency of these two alleles in the sequences generated in this study together with results from the PIRA-PCR assay, and the frequency in \( P. \) \( kuehnii \) sequences accessioned in the NCBI database, are given in Table 5. Allele −183A was observed in all 53 samples examined using the PIRA-PCR assay whereas −183G was observed only in 15 (52%) of the 29 samples from Australia, Japan, Philippines and China and absent from all samples obtained from the Western hemisphere. Similarly, −183G was absent from all sequences generated in this study and in the NCBI database from the Western hemisphere (Table 2). The detection frequencies of 183G:183A among samples from the Eastern hemisphere were 89% in the sequences generated in this study, 52% using the PIRA-PCR assay and 29% among sequences in the NCBI database.
Locked nucleic acid (LNA\textsuperscript{TM}) nucleosides were incorporated into the primers and probes used in the real-time assays. LNAs provide equivalent sensitivity and specificity as minor groove binding modifications (Letertre et al., 2003), by increasing the duplex melting temperatures. LNAs allow probes to be shorter (15–25 nucleotides) than unmodified probes which are typically 25–35 nucleotides. The probes developed in this study were up to half the length of fluorescent probes reported by Barnes & Szabo (2007) for four species of cereal rust pathogens. This feature is advantageous for detection methods based on variable sequences such as the ITS regions of rDNA since the portions of conserved sequence may be limited.

Since the first confirmed occurrence of \textit{P. kuehnii} in the Western hemisphere, the pathogen has been detected throughout Central America and the Caribbean basin. The sugarcane industries of Louisiana, Texas and South America, which so far remain free from infection, are under considerable threat from this pathogen. The \textit{P. kuehnii} assay developed in this study could be used for monitoring the spread of the pathogen in these regions. Quantitative real-time PCR has been used to monitor the spread of the soybean rust pathogen \textit{Phakopsora pachyrhizi} (Barnes et al., 2009) and several cereal rust pathogens (Barnes & Szabo, 2007) in rain collected in remote samplers. A similar strategy could be implemented for \textit{P. kuehnii} in the sugarcane growing regions of the Americas.

\textit{Puccinia kuehnii} and \textit{P. melanocephala} were detected in this study prior to uredinia development. Several previous studies have examined relationships between early detection of foliar pathogens and visual symptoms (Fraaije et al., 2001; Guo et al., 2006; Jackson et al., 2006; Lihua et al., 2008). This feature could prove useful for disease control strategies as it would allow control measures such as protective fungicides to be deployed, thereby limiting the spread of inoculum.

The PIRA-PCR assay developed in this study proved to be an effective method for detecting the SNP PkITS1 \textasciitilde183A\textasciitildeG in \textit{P. kuehnii}. This was not possible using hydrolysis probes (data not shown) possibly due to the A\textasciitildeT rich nature of the surrounding bases, and no restriction enzyme recognizing either of the SNP alleles could be identified.

One of the two SNP alleles was detected only in samples from Asia and Australia and was absent from all samples from the Caribbean basin tested in this study. The frequency of \textasciitilde183A and \textasciitilde183G varied with method of detection (sequencing, PIRA-PCR and NCBI accessioned sequences). This may reflect differences between the populations of \textit{P. kuehnii} isolates represented in each source. Alternatively this may be due to additional variations existing close to the 183A\textasciitildeG loci resulting in the non-detection of the two alleles by the PIRA-PCR assay.

Table 5  Frequency of the two alleles of a single nucleotide polymorphism identified in ITS1 of \textit{Puccinia kuehnii}. Data is separated by hemisphere of sample origin and method of detection: number of colonies sequenced, analysis of herbaria using PIRA-PCR assay and sequences accessioned in the NCBI database

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample detection</th>
<th>\textasciitilde183A</th>
<th>\textasciitilde183G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western</td>
<td>Sequences\textsuperscript{a}</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dried samples (PIRA-PCR)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NCBI</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Eastern</td>
<td>Sequences\textsuperscript{b}</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Dried samples (PIRA-PCR)</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>NCBI</td>
<td>24</td>
<td>7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Includes data for samples from USA, Guatemala, Nicaragua, Costa Rica, Mexico, Jamaica, Panama and El Salvador.
\textsuperscript{b}Includes data for samples from China, Philippines, Australia, Japan, Indonesia and Papua New Guinea.
\textsuperscript{c}From sequencing positive colonies from cloning reactions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Example of results from PIRA-PCR assay of \textit{Puccinia kuehnii} DNA. Lane 1: 20 bp ladder molecular marker; Lane 2: a 606 bp product amplified using PkPm primers; Lane 3: re-amplification of the PkPm product with primers PIRA-Pk and digested with the restriction enzyme \textit{BglII} (two bands are evident corresponding to the 136 bp undigested product (A allele) and the 109 bp digested product (G allele); Lane 4: the re-amplified, digested product for a sample which only contains the A allele.}
\end{figure}

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samples is that the allele was present at an extremely low frequency. However, this is unlikely since over 70 colonies were sequenced from samples collected in the Western hemisphere, as well as an additional 24 samples tested by PIRA-PCR.

Two forms of natural plant pathogen dispersal occur: single step invasions, where pathogen introduction occurs over a large area in a single event, and range expansions, where spread occurs gradually through incremental spore dispersal events (Brown & Hovmøller, 2002). _Puccinia melanocephala_ was found initially in the Dominican Republic following a single step invasion by spores carried from Cameroon by tropical weather systems. Range expansion of _P. melanocephala_ subsequently occurred rapidly through the Americas (Purdy et al., 1985).

Several possible mechanisms could explain the recent introduction of _P. kuehnii_ to the Western hemisphere. Although Asia and Australia were the only confirmed sources of _P. kuehnii_ before 2007, it is possible that the pathogen was initially aerially dispersed from this region to an intermediate location(s), remaining undetected or undiscovered due to confusion with the widely distributed brown rust, and subsequently spread to the Western hemisphere. Alternatively, unauthorized and improperly conducted movement of vegetative planting material which also contained _P. kuehnii_-infected leaves could have resulted in an initial spread of the pathogen from Asia or Australia followed by rapid and unnoticed spread. Recently, the rapid, global spread of the wheat yellow rust fungus _P. striiformis_ f. sp. _tritici_ has been reported (Hovmøller et al., 2008). The same strains of the pathogen that first appeared in North America in 2000 were detected in Western Australia just 2 years later. The spread of _P. kuehnii_ to the Western hemisphere could therefore have taken place in a similar, rapid and long distance dispersal event. Whichever mechanism led to the spread of _P. kuehnii_, only _–183A_ type isolates predominated in the Western hemisphere. This has important implications for the development of sugarcane cultivars with durable resistance to _P. kuehnii_. Sugarcane breeding for orange rust resistance must attempt to incorporate germplasm screening against isolates harbouring both _–183A_ and _–183G_ to combat any differences in virulence that may be associated with the two isolate types identified in this study.

**Acknowledgements**

The authors thank Kay McCorkle for technical assistance in the laboratory and Charles Barnes and Jerry Johnson at the Cereal Disease Laboratory for technical support and for providing _Puccinia_ spp. DNA.

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