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Pathogenic and molecular comparison of *Puccinia kuehnii* isolates and reactions of sugarcane varieties to orange rust

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Sugarcane orange rust, a disease caused by *Puccinia kuehnii*, was first reported in Brazil in 2009. There are no studies comparing the Brazilian *P. kuehnii* collections and the reaction of important sugarcane varieties under controlled conditions. This work compared the reaction of seven sugarcane varieties inoculated with six different *P. kuehnii* isolates from Brazilian sugarcane areas and verified the pathogenic and genetic variability of these isolates. The incubation (I) and latency (L) disease periods, disease severity (SEV), total number of lesions (TNL), total number of sporulating lesions (TNSL), and percentage of sporulating lesions (%SL) were evaluated. Furthermore, ITS1 and IGS ribosomal sequences of all *P. kuehnii* isolates used in this study were compared with pathogen sequences from 13 different countries. The disease incubation ranged from 7 to 10 days and the latency ranged from 10 to 21 days. SEV and TNL showed large variations and few significant differences between the reaction of the varieties to *P. kuehnii*, in contrast with the variables TNSL and %SL. The *P. kuehnii* isolates did not compose different virulent races, but the isolate from one site (Araras) was a more aggressive race. The ITS1 and IGS ribosomal sequences of six *P. kuehnii* isolates were identical with each other and to most *P. kuehnii* American sequences deposited at GenBank. The studied sequences of *P. kuehnii* isolates differed from the sequences from Asia, Tahiti and Oceania.

Keywords: orange rust, *Puccinia kuehnii*, *Saccharum* spp., sporulating lesions, sugarcane breeding

Introduction

Sugarcane orange rust (SOR), a disease caused by the fungus *Puccinia kuehnii*, was reported in Brazil in December 2009, in Sao Paulo State (Barbasso *et al.*, 2010). Previously, the pathogen had been reported in Central (Ovalle *et al.*, 2008) and North Americas (Comstock *et al.*, 2008), Asia and Oceania (Ryan & Egan, 1989), and Africa (Saumtally *et al.*, 2011). In Brazil, important sugarcane varieties such as SP89-1115, RB72-454 and SP84-2025 are susceptible to *P. kuehnii* (Barbasso *et al.*, 2010). The key strategy for disease control has been the use of genetic breeding (Magarey *et al.*, 2001; Berding *et al.*, 2004). Moreover, the sugarcane resistance to *P. kuehnii* may be considered a good example of polygenic resistance durability. In sugarcane, this resistance predominates and is the main source for breeding programmes to solve disease and pest problems (Robinson, 1987). However, in 2000, a break in resistance to *P. kuehnii* occurred in sugarcane variety Q124 in Australia, warning the sugarcane production areas around the world of the risks of rust-resistance breakdown.

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In Brazil, so far only three of the most planted varieties are considered susceptible to *P. kuehnii*. Nevertheless, at least 50 sugarcane varieties cultivated in the world are reported as susceptible or intermediately resistant (or 'moderately susceptible') to the pathogen. Furthermore, most pathogen resistance assessments are performed under field conditions, subject to environmental interferences (Purdy *et al.*, 1983; Taylor, 1992), affecting the genotypes' reaction to the pathogen (Camargo, 2011). Under controlled conditions, the sugarcane reaction to *P. kuehnii* may be different from the field observations. The different resistance levels of the varieties combined with variations between *P. kuehnii* isolates may lead to the appearance of pathogen isolates able to overcome the host resistance genes, as previously reported for some rusts (McDonald & Linde, 2002; Singh *et al.*, 2002). Since the 1970s, there have been reports of resistance breakdown in important sugarcane-producing areas (Berding *et al.*, 2004). In Florida (USA), high incidence levels of brown rust were reported in the variety CP79-1580 (previously classified as rust-resistant; Dean & Purdy, 1984). This was attributed to the emergence of new virulent races of *Puccinia melanocephala* against which the cultivated sugarcane varieties contained no source of resistance. An emergent race of *P. melanocephala* was also reported in the variety H56-7052 in USA (Comstock, 1988). Additionally, a study carried out under controlled conditions reported four

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pathogenic *P. melanocephala* races in Florida (Shine *et al.*, 2005). The breakdown in resistance to *P. kuehnii* in sugarcane variety Q124 in Australia in 2000 was supposedly caused by a new race of the pathogen (Braithwaite, 2005).

In Brazil, the extensive diversity in climate between sugarcane-producing areas combined with the genetic uniformity of the sugarcane grown (Robinson, 1987) has built favourable conditions for the rise of more aggressive races or variants of *P. kuehnii*. There are few studies that assess the reactions of different sugarcane varieties to the pathogen and compare, under controlled conditions, the *P. kuehnii* isolates from different sugarcane-producing areas. Therefore, it is necessary to understand better SOR in Brazil and the real risks of a further SOR epidemic. Thus, this study aimed to characterize the pathogenic and genetic diversity of *P. kuehnii* collections from different Brazilian sugarcane-producing areas, identify the possible new pathogen races and verify the reaction of sugarcane varieties to *P. kuehnii* isolates based on counting sporulating and nonsporulating lesions.

Materials and methods

Puccinia kuehnii isolates

Six isolates of *P. kuehnii* were collected: (i) AR, from an experimental sugarcane area planted with the variety SP81-3250 in the municipality of Araras, state of Sao Paulo; (ii) PR, from a commercial sugarcane area planted with the variety CT96-3415, in Paranacity, state of Parana; (iii) PI, from an experimental sugarcane area planted with the variety SP89-1115 in Piracicaba, state of Sao Paulo; (iv) RP, from a commercial sugarcane area also planted with the variety SP89-1115, in Ribeirao Preto, state of Sao Paulo; (v) NA, from a commercial sugarcane area planted with the variety RB72-454, in Nova Alvorada do Sul, state of Mato Grosso do Sul; and (vi) DO, from a commercial sugarcane area planted with the variety SP89-1115 in Dourados, state of Mato Grosso do Sul (Table S1). Prior to inoculation experiments, the cell wall of the collected urediniospores was analysed under an optical microscope to observe the typical apical thickening of *P. kuehnii* spores, which is not present in the cell wall of *P. melanocephala* urediniospores, causal agent of sugarcane brown rust.

Sugarcane varieties

Seven cultivated Brazilian sugarcane varieties with different levels of resistance to *P. kuehnii* (under field conditions) were inoculated with the six *P. kuehnii* isolates (Table 1). The plants were obtained from bud seed pieces and cultivated in pots of 700 mL capacity containing pine bark substrate. After planting, all plants received a single fertilization of ammonium sulphate (5 g per plant) and were grown in a greenhouse for 30 days. After 30 days, the plants were transferred to individual growth chambers for inoculations. Seven plants of each sugarcane variety were used in the experiments.

Puccinia kuehnii inoculation

The *P. kuehnii* inoculations were based on a methodology described by Martins *et al.* (2010): for all *P. kuehnii* inoculations, a urediniospore suspension of 10 mL was obtained by immersing sugarcane leaves with symptoms in distilled water and brushing them. The viability of urediniospores was checked 24 h before inoculation by a germination test in agar-water growth medium (Braithwaite *et al.*, 2009): 50 μL of the suspension of urediniospores (10^3 urediniospores mL^{-1} of distilled water) was incubated in a growth chamber in darkness at 25 °C for 8 h and then, the percentage of viable urediniospores (germinated) was calculated by observing 100 urediniospores. The percentage germination of urediniospores was used to standardize the inoculum (viable urediniospores) for each inoculation. For each of the six *P. kuehnii* isolates, the adaxial and abaxial surfaces of leaves of all sugarcane varieties were sprayed with a standard concentration of 5×10^4 viable urediniospores mL^{-1} of distilled water. The inoculations were repeated once using urediniospores produced from this first experiment, avoiding mixing of urediniospores. However, the amount of urediniospores collected from the first experiment was not enough to achieve the standard concentration of 5×10^4 viable urediniospores mL^{-1} of distilled water. Therefore, a correction factor (CF) was calculated to equate the two inoculations, dividing the concentration of urediniospores necessary to have 5×10^4 viable urediniospores mL^{-1} of distilled water by the concentration of the urediniospore suspension prepared from the first experiment. The CF was applied to the variables 'severity' (SEV), 'total number of lesions' (TNL), and 'total number of sporulating lesions' (TNSL).

The inoculated plants were kept in an incubation chamber under continuous darkness for 24 h. After this period, the plants that received the same *P. kuehnii* inoculum were maintained in individualized Conviron growth chambers at a temperature of 25 ± 2 °C until the end of the evaluations.

Table 1 Susceptibility level of variety (SLV) for seven sugarcane varieties inoculated with six *Puccinia kuehnii* isolates under field conditions.

Sugarcane variety	SLV	Reaction to *P. kuehnii*	References
SP89-1115	3	Susceptible	Barbasso *et al.* (2010)
RB85-5156	2	Intermediate	Minchio *et al.* (2011)
SP81-3250	2	Intermediate	Minchio *et al.* (2011)
RB86-7515	1	Resistant	Klosowski *et al.* (2013)
CTC 3	3	Susceptible	Nunes Junior (2010)
CTC 6	1	Resistant	Dalri (2012)
CTC 15	2	Intermediate	Dalri (2012)

Analysed variables

The following variables were evaluated: incubation period (I) – time interval (days) between inoculation and appearance of disease symptoms in at least 50% of the evaluated plants; latency period (L) – time interval (days) between inoculation and sporulation in at least 50% of evaluated plants with rust lesions; disease severity (SEV), quantified by estimating the percentage of leaf area with symptoms, based on the diagrammatic scale developed by Amorim *et al.* (1987); total number of lesions (TNL) and total number of sporulating lesions (TNSL), counted 21 days after inoculation (DAI) in a 20 cm leaf fragment from the most highly diseased leaf. In addition, TNSL/TNL was also calculated, resulting in the percentage of sporulating lesions (% SL). The evaluations were performed using a stereoscopic magnifying glass with ×40 magnification.

Phylogenetic analysis of *P. kuehnii* isolates

DNA extraction from P. kuehnii urediniospores

Urediniospores from each collection were homogenized for total DNA extraction, following the protocol developed by Dellaporta *et al.* (1983), with modifications: each sample was macerated in a 1.5 mL microtube containing 500 μL of extraction buffer (100 μM EDTA, 2.5 M ammonium acetate, 100 mM Tris buffer, pH 8.0). After maceration, 33 μL of a 20% SDS solution was added and the sample was homogenized and incubated in water at 65 °C for 10 min. After cooling, 160 μL of 5 M potassium acetate was added to the mix, which was centrifuged for 10 min at 23 269 ***g***. Subsequently, the aqueous phase was transferred to new microtubes containing 300 μL of isopropanol (4 °C) and was centrifuged at 23 269 ***g*** for 10 min. The supernatant was discarded, the resulting pellet was washed in 500 μL ethanol 75%, and the solution was centrifuged at 23 269 ***g*** for 5 min. The ethanol was discarded and the precipitate remained at room temperature to dry. Later, the pellet was resuspended in 50 μL of milli-Q water.

Polymerase chain reaction

Reactions were carried out in a total volume of 25 μL containing 30 ng *P. kuehnii* DNA, 1× PCR buffer, 3 mM $MgCl_2$, 200 μM dNTPs, 0.1 μM each oligonucleotide and 1.65 U *Taq* DNA polymerase.

The IGS region was amplified using the oligonucleotide pair LR12R/5SRNA (James *et al.*, 2001). The reaction conditions were as follows: initial preheating for 5 min at 95 °C; followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and a final cycle of 10 min at 72 °C. The ITS1 region was amplified using the oligonucleotide pair ITS2R (Braithwaite *et al.*, 2009)/ITS1F (Gardes & Bruns, 1993). The reaction conditions were as follows: initial preheating for 2 min at 95 °C; followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s, 72 °C for 80 s; and a final extension for 5 min at 72 °C. The products obtained from each PCR were electrophoresed in a 1.0% agarose gel and stained with ethidium bromide. The fragments corresponding to the genomic regions amplified were visualized in an ultraviolet transilluminator. A DNA sample of *P. kuehnii* maintained by the Sugarcane Research Centre (CTC) was used as a positive control. PCR products were sequenced in the Biotechnology Laboratory of ESALQ/USP in Piracicaba, state of Sao Paulo, Brazil.

The six nucleotide sequences of the *P. kuehnii* isolates were submitted to GenBank (accession numbers KY024484 to KY024495), compared with each other, and with those previously deposited in GenBank (NCBI) (27 for IGS and 51 for ITS1) to determine the genetic similarity level between them (Table S1). All sequences were aligned by the MUSCLE method (Edgar, 2004) using the MEGA v. 5.0 software (Tamura *et al.*, 2011). Gaps were considered as lost data and terminal regions with dubious alignment were removed manually using BioEdit v. 7.1.11. All *P. kuehnii* sequences are made available as Files S1, S2 and S3.

Phylogenetic analysis was performed using the Bayesian inference method with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001). The optimal model was obtained with MrModeltest v. 2.3, with AIC and Kimura 3 using default settings. Analyses of Bayesian inference were performed with a random starting tree and Markov chain Monte Carlo (MCMC) for 10^6 generations. Sequences of ITS1 and IGS from *P. melanocephala* (FJ009328 and FJ009329, respectively) were included as out-group in order to compare the two sugarcane rust pathogens and for rooting the phylogenetic trees. *Puccinia melanocephala* was chosen as the out-group as it represents the first and deepest split between sugarcane rusts; both *Puccinia* species have several similar morphological characteristics but are genetically different. Sequences of *P. melanocephala* were also used in another similar study (Braithwaite *et al.*, 2009) as a root for phylogenetic trees. An analysis of Bayesian inference was performed with partitioned data of ITS and IGS. The generated phylogenetic trees were edited by FIGTREE v. 3.1 2006-2009.

Data analysis

SEV, TNL, TNSL and %SL data were submitted to analysis of variance followed by the Scott–Knott comparative test of means at 5% significance. Having applied the Scott–Knott test, data of the variables SEV, TNL and TNSL were transformed by square root of $(x + 1)$. For each sugarcane variety used in this study, a susceptibility level was assigned (SLV) based on the susceptibility level of sugarcane varieties to *P. kuehnii* under field conditions: grades 3, 2 and 1 for susceptible, intermediate and resistant varieties, respectively (Table 1). Once the SLV for each sugarcane variety was determined, a correlation analysis was performed between the variables SLV, SEV, TNL, TNSL and % SL. Furthermore, rankings of *P. kuehnii* isolates were built based on the results of the Scott–Knott test applied for the variables SEV, TNL and %SL.

Results

Incubation and latency period

The incubation period ranged from 7 to 10 days in both experiments without significant differences between the varieties, regardless of SLV. The latency period ranged from 10 to 21 days. In the susceptible varieties SP89-1115 and CTC 3, latency ranged from 10 to 12 days. Sporulating lesions were not observed in the resistant varieties RB86-7515 and CTC 6 during the assessment period (21 days).

Disease severity and total number of lesions

The lowest average SEV was observed in the variety RB86-7515, considered resistant to *P. kuehnii*, inoculated with the isolate PI (Table 2). The sugarcane varieties CTC 3 and SP89-1115 showed the highest disease severity, except when inoculated with isolates AR and NA. When inoculated with isolates AR, PR, NA and DO, even the resistant varieties, such as CTC 6, and the intermediate varieties CTC 15, RB85-5156 and SP81-3250 showed severity levels significantly equal to those considered susceptible. For the SLVs 1, 2 and 3, the SOR severity averages ranged from 0.92% to 5.80%, 1.53% to 11.39%, and 3.28% to 11.83%, respectively (Table 2). The correlation between SLV and SOR severity was 0.04.

The average TNL (sporulating + nonsporulating lesions) ranged from 75.93 (isolate PI on sugarcane variety RB85-5156) to 742.81 (isolate AR on sugarcane variety SP81-3250) (Table 2). For the SLVs 1, 2 and 3, the average TNL ranged from 84.32 to 530.11, 75.93 to 742.81, and 135.05 to 528.43, respectively. Major

Table 2 Sugarcane orange rust severity and total number of lesions of the six *Puccinia kuehnii* isolates on seven sugarcane varieties used in this study.

	Collection location[a]											
	PI		AR		RP		PR		NA		DO	
Sugarcane variety	SEV[b]	TNL[c]	SEV	TNL	SEV	TNL	SEV	TNL	SEV	TNL	SEV	TNL
SP89-1115	3.29 aB	135.05 aB	5.65 bB	393.45 cA	4.09 bB	226.78 aB	11.83 aA	320.43 aA	6.65 bB	154.27 bB	4.62 bB	191.69 bB
RB85-5156	1.53 bC	75.93 bC	4.90 bB	289.21 cA	3.24 bC	93.30 bC	8.04 aA	330.46 aA	4.69 cB	156.61 bB	3.02 bC	145.48 bB
SP81-3250	2.04 bB	92.11 bD	11.39 aA	742.81 aA	3.53 bB	120.16 bD	8.14 aA	338.87 aB	10.37 aA	291.31 aB	4.52 bB	203.33 bC
RB86-7515	0.92 bA	84.32 bD	2.53 cA	530.11 bA	1.20 bA	84.60 bD	2.17 bA	292.94 aB	2.85 cA	162.76 bC	2.67 bA	157.52 bC
CTC 3	3.28 aB	165.35 aD	8.83 aA	528.43 bA	8.91 aA	250.09 aC	7.95 aA	335.98 aB	5.41 bB	143.24 bD	7.69 aA	321.78 aB
CTC 6	1.38 bB	118.64 aC	3.53 cB	505.27 bA	3.43 bB	184.33 aC	5.78 aA	297.22 aB	3.10 cB	144.34 bC	5.80 aA	326.16 aB
CTC 15	1.64 bB	115.04 bC	3.54 cA	314.38 cA	1.72 bB	121.08 bC	6.45 aA	329.98 aA	2.65 cB	84.27 cC	3.82 bA	193.62 bB

For each variable, values at the same line followed by identical uppercase letters and values at the same column followed by identical lowercase letters do not differ at 5% by Scott–Knott test. Data transformed by square root of $(x + 1)$ for statistical analysis.

[a]Isolates were collected from the following locations: PI, Piracicaba, state of Sao Paulo; AR, Araras, state of Sao Paulo; RP, Ribeirao Preto, state of Sao Paulo; PR, Paranacity, state of Parana; NA, Nova Alvorada do Sul, state of Mato Grosso do Sul; DO, Dourados, state of Mato Grosso do Sul.

[b]SEV, disease severity quantified by estimating the percentage of leaf area with symptoms, based on the diagrammatic scale developed by Amorim *et al.* (1987).

[c]TNL, total number of lesions, both sporulating and nonsporulating.

differences between sugarcane varieties were observed after inoculations with AR and NA, where the TNL was significantly higher in the sugarcane variety SP81-3250 than in the other varieties. In the sugarcane varieties SP89-1115, RB85-5156 and CTC 15, the TNL was significantly higher after inoculation with AR and PR than with other isolates (Table 2). The correlation between TNL and SLV was low: 0.01.

The *P. kuehnii* ranking showed significant differences, regardless of *P. kuehnii* isolate and sugarcane variety used (Table 3). The TNL ranking was also significant and indicated that the largest TNL in all sugarcane varieties was observed after inoculation with AR, followed by PR. The least TNL was reported with isolates RP and PI (Table 3).

Total number of sporulating lesions and percentage of sporulating lesions

The TNSL and %SL showed significant differences between the sugarcane varieties and *P. kuehnii* isolates (Table 4). The resistant varieties (SLV = 1) RB86-7515 and CTC 6 showed sporulating lesions only when inoculated with AR (0.94%SL on RB86-7515) or AR, PI and DO (1.25, 0.31 and 1.36%SL, respectively, on CTC 6). After inoculation with other *P. kuehnii* isolates, these sugarcane varieties had no sporulating lesions by 21 DAI (Table 4). The highest TNSL in the resistant varieties was 6.99 (AR on variety CTC 6). The TNSL of the intermediate varieties (SLV = 2) RB85-5156, SP81-3250 and CTC 15 ranged from 2.61 (RP variety SP81-3250) to 660.15 (AR on variety SP81-3250), corresponding to 1.13% and 88.29%SL, respectively. The TNSL in the susceptible sugarcane varieties (SLV = 3) SP89-1115 and CTC 3 ranged from 121.42 to 502.56, and from 84.39% to 97.91%SL (Table 4). The correlations between TNSL and SLV, and between SLV and %SL were 0.56 and 0.75, respectively. The %SL ranking showed significant differences between *P. kuehnii* isolates by Scott–Knott test at 5% of significance. The highest % SL occurred with AR, regardless of the inoculated sugarcane variety (Table 3).

Phylogenetic analyses of *P. kuehnii* isolates

Five main groups of isolates were identified by the ITS region, three by the IGS, and seven in the concatenated tree. The six Brazilian *P. kuehnii* isolates were placed in the largest group for each tree, along with *P. kuehnii* isolates from Australia, Indonesia, Cuba (ITS1, IGS and concatenated tree), China (ITS1 and IGS), Papua New Guinea, Philippines, Japan, Guatemala, Panama, Jamaica, Costa Rica, Nicaragua, Mexico, Argentina, Guyana and United States (ITS1). The posterior probability values for the major clades of ITS1 ranged from 0.54 to 1.00 (Fig. 1), from 0.99 to 1.00 for IGS (Fig. 2), and from 0.58 to 1.00 for the concatenated tree (Fig. 3).

The isolates BPI79612 (Australia) and BPI7964 (Tahiti), collected in 1935 and 1916, respectively,

Table 3 Ranking of disease severity, total number of lesions and percentage of sporulating lesions in the sugarcane varieties SP89-1115, CTC3, RB85-5156, SP81-3250, CTC15, RB86-7515 and CTC6 inoculated with *Puccinia kuehniii* isolates.

P. kuehnii isolate[a]	SP89-1115	CTC 3	RB85-5156	SP81-3250	CTC 15	RB86-7515	CTC 6
Disease severity[b]							
AR	2	1	2	1	1	1	2
RP	2	1	3	2	2	1	2
PI	2	2	3	2	2	1	2
PR	1	1	1	1	1	1	1
NA	2	2	2	1	2	1	2
DO	2	1	3	2	1	1	1
Total number of lesions[b]							
AR	1	1	1	1	1	1	1
RP	2	3	3	4	3	4	3
PI	2	4	3	4	3	4	3
PR	1	2	1	2	1	2	2
NA	2	4	2	2	3	3	3
DO	2	2	2	3	2	3	2
Percentage of sporulating lesions							
AR	1	1	1	1	1	1	1
RP	1	1	4	4	3	1	1
PI	1	1	3	3	2	1	1
PR	1	1	1	4	1	1	1
NA	1	2	3	3	3	1	1
DO	1	2	2	2	2	1	1

Ranking based on the results of the Scott–Knott test applied for the variables disease severity, total number of lesions, and percentage of sporulating lesions, where 1 = most resistant and 4 = most susceptible.

[a]AR, Araras; RP, Ribeirao Preto; PI, Piracicaba; PR, Paranacity; NA, Nova Alvorada do Sul; DO, Dourados.

[b]Data transformed by square root of ($x + 1$).

composed a very distinct clade of the ITS tree. In this tree, all *P. kuehnii* isolates from the American continent were grouped in one clade, except for the isolates BPI878289-United States (2007) and Pk Misiones Arg-Argentina (2015) that grouped into a separate clade (Fig. 1, clade 2). In contrast, isolates from Asia and Oceania were present in all defined clades, irrespective of the year of collection. Isolate BRIP45350 from Indonesia was more distantly related to the others in the same cluster (Fig. 1). For IGS, most of the *P. kuehnii* isolates from Australia were in group 1, along with the six Brazilian isolates used in this study. *Puccinia kuehnii* isolates from Indonesia were grouped in all three main clades and isolates from Papua New Guinea grouped into the two smaller clades (Fig. 2, clades 2 and 3). The concatenated tree showed seven main clades. Group 1 (major clade) encompassed the six Brazilian isolates and isolates from Australia, Indonesia and Cuba. The other clades contained *P. kuehnii* isolates from Indonesia, China, Papua New Guinea, Samoa and Australia obtained from 1961 onwards (Fig. 3). The isolates BRIP47011, BRIP47039 and BRIP39604 from Indonesia each composed a

Table 4 Total number of sporulating lesions (TNSL) and percentage of sporulating lesions (%SL) at 21 days after the inoculation of seven sugarcane varieties with six *Puccinia kuehnii* isolates.

	Collection location[a]											
	PI		AR		RP		PR		NA		DO	
Sugarcane variety	TNSL	%SL	TNSL	%SL	TNSL	%SL	TNSL	%SL	TNSL	%SL	TNSL	%SL
SP89-1115	150.20 aB	97.91 aA	374.38 cA	95.27 aA	202.42 aB	90.52 aA	310.87 aA	96.95 aA	131.47 aB	92.44 aA	175.27 bB	90.59 aA
RB85-5156	23.12 cC	31.53 cC	195.49 dA	67.06 cA	14.94 bC	16.96 cD	239.10 aA	67.70 cA	68.15 bB	37.91 cC	85.40 cB	54.24 cB
SP81-3250	18.71 cC	22.29 dC	660.15 aA	88.29 bA	2.61 cC	1.13 dD	2.72 bC	0.89 D	86.63 bB	24.88 dC	85.91 cB	44.89 dB
RB86-7515	0.00 dA	0.00 eA	5.47 eA	0.94 dA	0.00 cA	0.00 dA	0.00 bA	0.00 dA	0.00 cA	0.00 eA	0.00 dA	0.00 eA
CTC 3	159.88 aD	95.80 aA	502.56 bA	95.54 aA	233.21 aC	92.80 aA	320.59 aB	95.40 aA	121.42 aD	84.39 bB	280.35 aC	84.78 aB
CTC 6	0.07 dA	0.31 eA	6.99 eA	1.25 dA	0.00 cA	0.00 dA	0.00 bA	0.00 dA	0.00 cA	0.00 eA	2.85 dA	1.36 eA
CTC 15	74.29 bC	60.96 bB	226.91 dA	72.06 cA	37.31 bD	29.22 bC	216.16 aA	78.31 bA	29.93 bD	30.26 dC	123.41 bB	64.12 bB

For each variable, values in the same line followed by identical uppercase letters, and values in the same column followed by identical lowercase letters do not differ at 5% by Scott–Knott test. TNSL data transformed by square root of ($x + 1$) for statistical analysis.

[a]Isolates were collected from the following locations: PI, Piracicaba, state of Sao Paulo; AR, Araras, state of Sao Paulo; RP, Ribeirao Preto, state of Sao Paulo; PR, Paranacity, state of Parana; NA, Nova Alvorada do Sul, state of Mato Grosso do Sul; DO, Dourados, state of Mato Grosso do Sul.

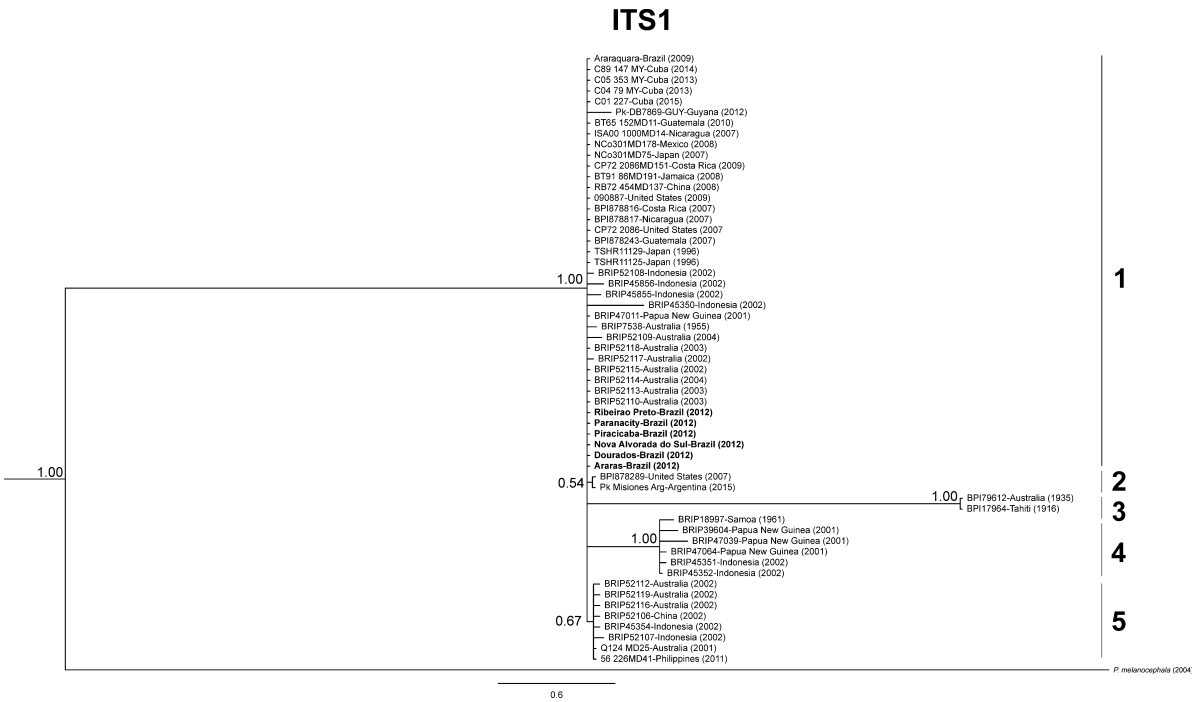

Figure 1 Phylogenetic tree of Bayesian analysis of ITS1 region of *Puccinia kuehnii*, rooted with *Puccinia melanocephala* (FJ009328). The support for each of the major clades (posterior probability) is indicated next to the most relevant nodes.

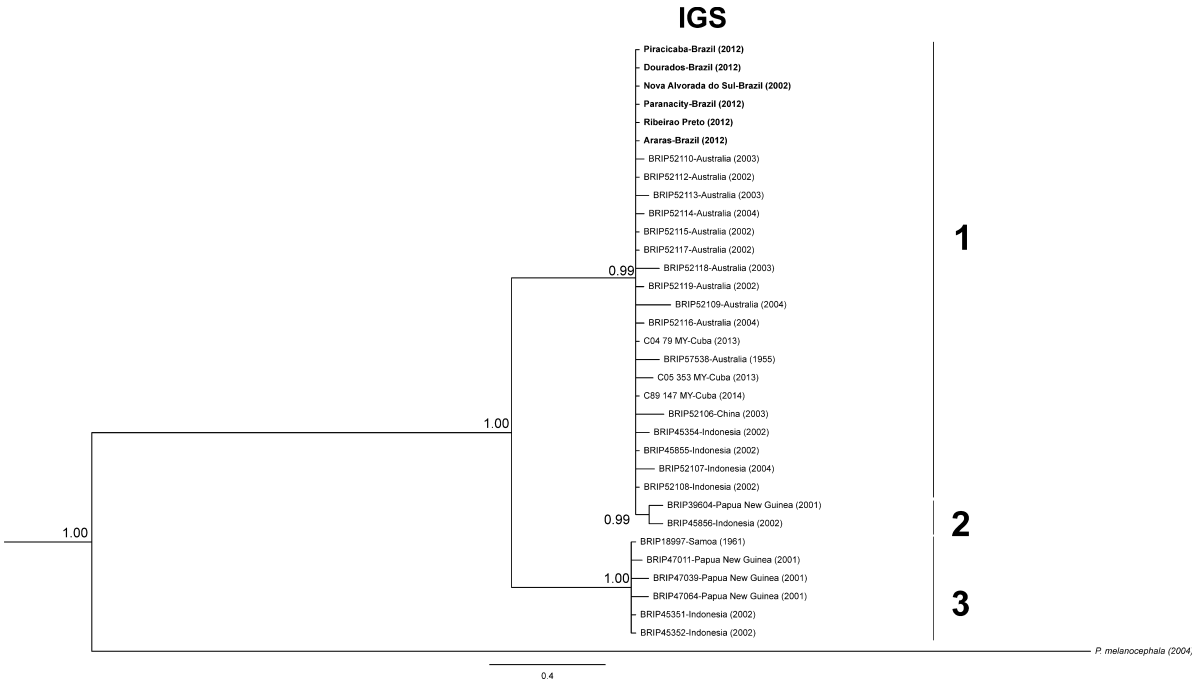

Figure 2 Phylogenetic tree of Bayesian analysis of IGS region of *Puccinia kuehnii*, rooted with *Puccinia melanocephala* (FJ009329). The support for each of the major clades (posterior probability) is indicated next to the most relevant nodes.

separate clade (Fig. 3, clades 3, 5 and 7, respectively). Other small clades (4 and 6) encompassed *P. kuehnii* isolates from Samoa, Indonesia and Papua New Guinea (Fig. 3).

Discussion

The incubation period of *P. kuehnii* provided little information on the degree of resistance of sugarcane varieties

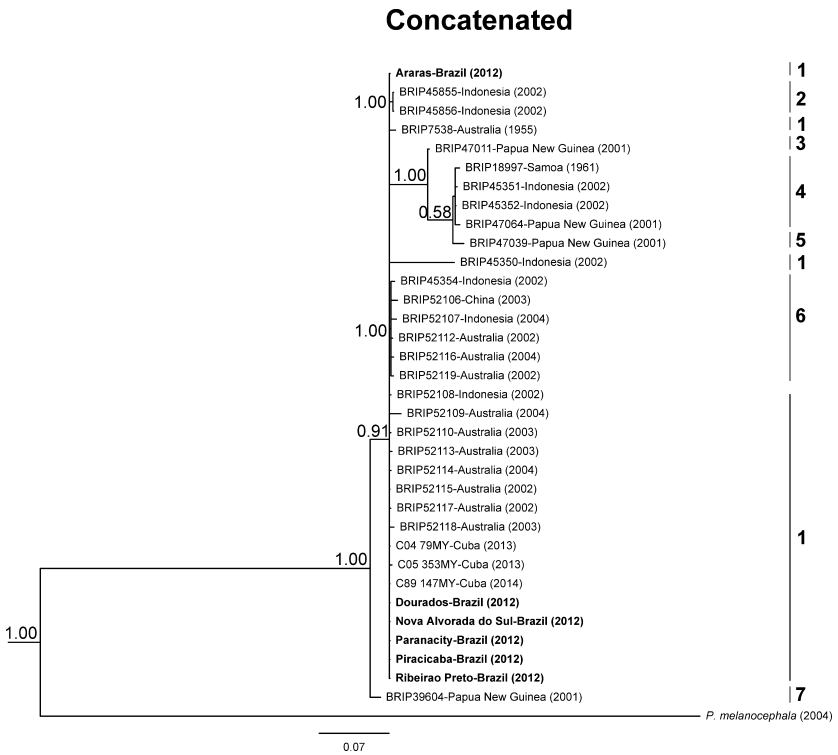

Figure 3 Concatenated tree of Bayesian analysis of ITS1 and IGS region of *Puccinia kuehnii*, rooted with *Puccinia melanocephala* (FJ009329). The support for each of the major clades (posterior probability) is indicated next to the most relevant nodes.

to this pathogen, as the range (from 7 to 10 days) was insufficient to provide significant differences between varieties. The results indicated that the components of sugarcane disease resistance do not influence the time for symptom expression. In other pathosystems involving the genus *Puccinia*, such as *Puccinia arachidis* on peanut, incubation periods were longer on more resistant genotypes than on intermediate and susceptible genotypes (Subrahmanyam *et al.*, 1983). In the *Puccinia triticina*–wheat pathosystem, incubation periods were short on susceptible varieties and long on resistant varieties (Sareen *et al.*, 2012).

The latency period on the sugarcane varieties ranged from 10 to 21 days. Latency periods were short (10 to 12 days) on susceptible varieties SP89-1115 and CTC 3, but 50% sporulation of lesions was not observed on the resistant varieties RB86-7515 and CTC 6 during the 21 days of the study. These results indicate that latency period is a resistance component strongly expressed in the resistant sugarcane varieties. In the intermediate resistance variety SP81-3250, latency was similar to susceptible varieties for AR (11 days), but the intermediate varieties RB85-5156 and CTC 15 showed latency of 16 and 21 days, respectively. Resistance is considered low when latency and incubation periods are short and high when such periods are long (Kranz, 2002). Generally, incubation and latency periods are sensitive to changes in temperature, quantitative resistance level, plant age, inoculum density and nutritional status (Kranz, 2002). In the present study, the difference between incubation period and latency period was large, indicating a poor correlation between these variables. Therefore, it is recommended that the latency period is measured in assessments of sugarcane genotypes in breeding programmes, because the determination and use of this variable are relatively easy and fast (Parlevliet *et al.*, 1980; Broers & Jacobs, 1989; Roumen, 1996).

The SOR severity, even though quite variable between the experiments, was low in resistant sugarcane varieties, especially in RB86-7515 inoculated with PI. Not surprisingly, susceptible varieties showed the highest disease levels in most inoculations. However, the levels of SOR severity on intermediate varieties, and even the resistant variety CTC 6, were significantly equal to the severity levels shown by susceptible varieties, mainly in AR, PR and DO inoculations. Results of disease severity and incubation period, if considered alone, do not clearly distinguish the differences between *P. kuehnii* isolates and the susceptibilities of sugarcane varieties (low correlation between SEV and SLV: 0.04).

Variations between isolates and varieties were also observed for TNL. In addition to showing a short latent period, the variety SP81-3250 showed a large TNL when inoculated with AR. This indicates that AR may belong to a more aggressive race of *P. kuehnii*. However, the TNL cannot be used as a variable to rank the resistance of sugarcane genotypes to *P. kuehnii* because even the resistant varieties such as RB86-7515 and CTC 6 showed

high TNL, causing a low correlation (0.01) with the SLV.

The variables most suitable for classifying the relative resistance of sugarcane varieties to *P. kuehnii* and aggressiveness of isolates were found to be TNSL and %SL; the high aggressiveness of AR was unmistakable using these variables, especially in the variety SP81-3250, which showed almost 90% SL after inoculation with AR. Susceptible varieties, such as SP89-1115 and CTC 3, showed high %SL (over 84%). In contrast, the resistant varieties RB86-7515 and CTC 6 showed %SL below 1.5% or even no sporulating lesions during the evaluation period. The largest range of %SL occurred in the intermediate varieties (0.89–78.31%). The results from %SL were more consistent when associated with TNSL; both %SL and TNSL showed a good correlation with SLV, of 0.75 and 0.56, respectively. Thus, the results indicate that the combined use of TNSL and % SL variables is suitable for distinguishing susceptible sugarcane varieties from those that are resistant to *P. kuehnii* and would be a useful tool in breeding programmes.

The races of a pathogen correspond to variations within the same species, defined by the spectrum of actions of the pathogen against a set of varieties (Camargo, 2011). These variations include two types of pathogen races: aggressive and virulent (Vanderplank, 1968). The rise of a new virulent race takes place when there is a mutant pathogen genotype able to break the variety resistance (Camargo, 2011) and there are obvious, large differential interactions between different pathogen isolates and different host genotypes (Vanderplank, 1968; Robinson, 1987). Conversely, pathogen races that do not interact differently with host genotypes, but vary in their aggressiveness, correspond to aggressive races (Vanderplank, 1968). Considering these concepts, the variables evaluated in the present study were not sufficient to detect a new virulent race of *P. kuehnii* in Brazil from the six isolates investigated. The differences between the interactions of these isolates with the sugarcane varieties were minimal and probably inherent to the experimental deviations or even to the action genes with small effects present in the sugarcane varieties used. However, the pathogenic differences found between the six *P. kuehnii* isolates were sufficient to identify a more aggressive race of the pathogen in Brazil, collected in Araras, Sao Paulo (AR), which caused greater TNL and % SL, regardless of the sugarcane variety.

The molecular analysis of the ITS and IGS sequences and a combination of them, showed no differences between the six *P. kuehnii* isolates used in this study. These regions are conserved and represent only a small fragment of the pathogen genome. Previous studies showed that variations within this region may be insufficient to distinguish the virulence of *P. kuehnii* isolates (Braithwaite *et al.*, 2009). Nevertheless, the *P. kuehnii* phylogenetic trees constructed in the present study revealed five clusters using the ITS region, three using the IGS region and seven using the concatenated sequences. The three phylogenetic trees suggest differences in time and space between the *P. kuehnii* isolates. In the tree based on the IGS region, the six *P. kuehnii* isolates collected in Brazil and those collected in Australia after the 2001 outbreak (Magarey *et al.*, 2001) were grouped in the largest group. Braithwaite *et al.* (2009) argue that the only rare presence of *P. kuehnii* teliospores (Virtudazo *et al.*, 2001) prevents the occurrence of sexual recombination. Therefore, the authors suggest that the sequenced IGS region of the *P. kuehnii* Australian populations represent a single dominant genotype. This argument can also be extended to the Brazilian *P. kuehnii* isolates used in this study.

The break of resistance in the variety Q124 in 2000 in Australia may have been caused by a simple mutation, producing a new *P. kuehnii* race that would have spread quickly across the country (Braithwaite *et al.*, 2009). Indeed, in the tree based on the ITS region presented here, the Australian isolate BPI79612, collected in 1935, was separated from the others Australian isolates collected since the epidemic in 2000. However, the likelihood of these differences on the ITS phylogenetic tree being due to a possible accelerated rate of molecular evolution in the *P. kuehnii* isolates is low because they belong to conserved regions from DNA. Nevertheless, distant clusters (as found for the ITS region) may be an important representation of groups that were more diverse. *Puccinia kuehnii* isolates BRIP47011, BRIP47039 and BRIP39604 were in groups with other isolates in the ITS1 and IGS trees, but were positioned in isolated clades in the tree of concatenated sequences. Other minor variations among isolates are most likely to be a result of PCR errors (Braithwaite *et al.*, 2009). The *P. kuehnii* isolates from Australia, Papua New Guinea and Indonesia, central sources of the pathogen, are present in most clusters of the three phylogenetic trees, indicating possible pathogen variations over time and space. In the ITS1 region, all Brazilian and most Australian *P. kuehnii* isolates were located in the largest cluster. As already reported by Braithwaite *et al.* (2009), the isolates from Indonesia indicate greater diversity by their presence in the largest groups from the three trees. Nearly all isolates collected in North, Central and South America are in the same clade, in agreement with the results of Glynn *et al.* (2010) who analysed this same rDNA region from *P. kuehnii*. The only exceptions were isolates BPI878289-United States (2007) and Pk Misiones Arg-Argentina (2015), which grouped into a separate clade. All trees showed isolates from Indonesia or Papua New Guinea present in all clusters and isolates from Central America (i.e. Cuba) were grouped together with Brazilian isolates.

Overall, the high genetic similarity of the *P. kuehnii* isolates and the low pathogenic variability between pathogen isolates located in the Central-South sugarcane area, combined with the reliability of variables such as the %SL and the latency period, will contribute to the selection of genotypes with resistance to SOR in sugarcane breeding programmes in Brazil. In particular,

breeding programmes focused on polygenic resistance should provide a more effective, lasting, and predominant resistance in the sugarcane crop. Finally, it has been shown that the *P. kuehnii* isolate from Araras, Sao Paulo, is from an aggressive race of the pathogen.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Isolates of *Puccinia kuehnii* and *Puccinia melanocephala* used for phylogenetic analyses of rDNA regions IGS and ITS1.

File S1. *Puccinia kuehnii* sequences used for phylogenetic analysis of the ITS region.

File S2. *Puccinia kuehnii* sequences used for phylogenetic analysis of the IGS region.

File S3. *Puccinia kuehnii* sequences used for concatenated phylogenetic analysis of the ITS and IGS regions.