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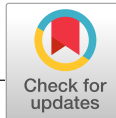
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Genetic diversity of *Puccinia kuehnii*, the causal agent of orange rust of sugarcane, from Brazil

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Abstract

The use of resistant genotypes is the preferred method to control orange rust of sugarcane (*Saccharum* spp) caused by *Puccinia kuehnii*. This approach has been adopted in Brazil but outbreaks of the disease on previously resistant varieties showed that the efficacy of this method is limited and requires a better understanding of pathogen diversity. Nevertheless, adequate molecular markers for examining pathogen diversity at population level are not available, which limits the success of orange rust control by genetic resistance. Therefore, two independent investigations were conducted to examine genetic diversity of *P. kuehnii* from São Paulo state, the most important sugarcane growing state of Brazil. First, simple-sequence repeat (SSR) markers were developed in the present work and genotypic diversity of orange rust isolates from different locations investigated. Second, phenotypic diversity was examined by the single-pustule inoculation technique on *P. kuehnii* isolates retrieved from three susceptible commercial sugarcane cultivars. A total of 96 SSR markers were generated and tested for this species. Subsequently, 29 isolates of *P. kuehnii* were fingerprinted with nine SSR markers to estimate the genotypic diversity by neighbour-joining and 3D principal coordinates. The 29 isolates of the pathogen clustered into four main groups, which were identified by three SSR markers (NPRL_PK_108a, NPRL_PK_162_spka and NPRL_PK_221_spka). Phenotypic data at 21 days after the single-pustule inoculation showed that *P. kuehnii* from highly susceptible commercial cultivars harboured a small proportion of variants capable of causing disease on resistant cultivars. A differential reaction was demonstrated for the most virulent variant in a repeated experiment confirming the existence of races within *P. kuehnii* in Brazil.

KEYWORDS

genotypic, inoculation method, phenotypic, races, *Saccharum*, SSR

Part of MSc dissertation by T. F. Mistura at PPGPVBA/UFSCar

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1 | INTRODUCTION

Orange rust, caused by the basidiomycete *Puccinia kuehnii* [(W. Kruger) E.J. Butler], was first detected in Brazil in São Paulo state in December 2009 (Barbasso et al., 2010). The first recommendation to cope with this disease was the discard and replacement of susceptible cultivars: SP89-1115, RB72454 and SP84-2025 (Barbasso et al., 2010). This strategy was adopted with success by the Australian sugarcane industry to control the first known epidemic of the orange rust, which occurred on cultivar Q124 in early 2000 (Magarey et al., 2001). Therefore, the outbreaks of the orange rust observed on commercial cultivars SP81-3250 and CTC15 in 2012/2013 (Raizen, 2013) were unexpected, since they were not included as susceptible before. Nevertheless, it is unknown whether their resistance was broken or they were always susceptible because they were not mentioned by the time of the first outbreak. Later studies showed uncertain results, one indicated both as moderately resistant (Araújo et al., 2013) and the other, SP81-3250 as moderately susceptible (Chapola et al., 2016). The reason why the method of control applied in Australia did not work likewise in Brazil is still unknown.

The success of disease control by plant genotype resistance depends on the knowledge of pathogen diversity (Chen et al., 2006). In pathogens that undergo continuous life cycles of asexual reproduction, such as *Puccinia*, changes in genetic diversity are more prone to occur (Anderson et al., 2010). Therefore, an effective breeding programme requires a comprehensive information on genetic diversity of the pathogen (Pocovi et al., 2010).

Genetic diversity can be examined by genotypic and phenotypic traits. When *P. kuehnii* from Queensland and New South Wales in Australia were investigated by sequencing of ITS region, a low variation among 28 field isolates was detected, while greater sequence diversity occurred within rust collection from other countries (Indonesia, Papua New Guinea, China) (Braithwaite et al., 2009). Subsequently, sequencing of the ITS region revealed that the Caribbean population possessed only the 183A allele, whereas Australian and Asian populations showed a further allele (183G), suggesting differences between orange rust populations from both continents (Glynn et al., 2010). The same ITS region did not discriminate within country the diversity among isolates of *P. kuehnii* from Brazil (Moreira et al., 2018). Not surprisingly, the ITS region is not adequate to examine diversity at population level, since they are highly conserved intraspecifically, variation occurs among different species (Bruns et al., 1991). Variation at population level or closely related individuals should make use of markers derived from fast changing regions, such as SSR (simple-sequence repeats), due to high mutation rate affecting the number of repeats and making them highly polymorphic (Abdel-Mawgood, 2012). Because such markers are not available for *P. kuehnii*, information on genotypic diversity at population level has not been clarified.

Phenotypic diversity of *P. kuehnii* carried out by the time of the first outbreak of orange rust in Australia suggested that a change in the race of the pathogen was the cause of the epidemic of 2000 (Magarey et al., 2001). Similarly, change in pathogenicity was

suggested for the orange rust outbreak on CP 88-1762 in Florida, a cultivar that had not shown symptoms previously (Comstock et al., 2010). On the other hand, reason for the orange rust outbreaks in Brazil on SP81-3250 and CTC15 in 2012/13 (Raizen, 2013) is still a matter of controversy, since different fungal races were attributed by some researchers (Urashima et al., 2014) while difference in aggressiveness and not races, by others (Moreira et al., 2018). Fungal races are defined as the existence of significant differential reaction to orange rust between two isolates of *P. kuehnii* towards a sugarcane cultivar (Shine et al., 2005). A robust research with larger number of rust isolates and accurate inoculation method would reveal existence or not of fungal races, vital for the orange rust control by resistant genotypes.

Therefore, to investigate the genetic diversity of *P. kuehnii* two independent investigations were carried out: first, genotypic diversity of orange rust isolates collected from various geographic locations of São Paulo state, the most important of Brazil, was investigated by SSR markers developed in the present work. Second, phenotypic diversity was examined by single-pustule inoculation technique on *P. kuehnii* retrieved from three susceptible commercial sugarcane cultivars.

2 | MATERIALS AND METHODS

2.1 | 2.1. Genotypic diversity

2.1.1 | SSR marker development

Isolates of *P. kuehnii* were collected from leaves of sugarcane cultivars exhibiting suspected symptoms of orange rust. Subsequently, the causal agent of the orange rust was confirmed in laboratory by light microscopy based on reported morphological traits of urediniospores: orange colour, ellipsoidal format, surrounded by echinulate ornamentation and thickened apical wall (Comstock et al., 2008). Total DNA was extracted from pustules of orange rust according to the procedure described by Murray and Thompson (1980). Sporulating uredinial lesions were selected and cut in pieces to fill half of a 2-ml microtube, incubated at 65°C in CTAB buffer, centrifuged, precipitated and washed (Crous et al., 2009). Simple-sequence repeat-enriched libraries were generated by subtraction hybridization using multiple biotinylated oligo repeats and linker: SSRLIBF3: 5'-CGGGAGAGCAAGGAAGGAGT-3' and SSRLIBR3: 5'-Phos-CTCCTTCCTTGCTCTCTCCCGAAAA-3' as previously reported (Tehen et al., 2010). Fragments containing SSRs were cloned into TOPO4 vector (Invitrogen, CA) and sequenced with chain-terminating inhibitors (Sanger et al., 1977). Sequences were assembled using DNASTAR Lasergene 10 (DNASTAR, Inc.) and screened for potential presence of plant DNA using BLAST analysis (Altschul et al., 1990). Detection of SSRs was performed with SSR Finder (Sharopova et al., 2002) and Sputnik (available from Chris Abajian, University of Washington, WA, USA; chrisa@espresso-software.com) scripts. Primers were designed using Primer3 (Rozen & Skaletsky, 2000).

2.1.2 | Genotyping of *P. kuehnii* isolates

A total of 96 primer sets were designed and tested by DNA fingerprinting on four rust isolates, two of orange rust (*P. kuehnii*) and two of brown rust (*P. melanocephala*). Fingerprinting of these four isolates was performed as reported before (Arias et al., 2011), and amplicons were analysed using GeneMapper v. 4.0 (Applied Biosystems). These SSR markers were employed to examine the genotypic diversity of 29 isolates obtained from Brazilian sugarcane cultivars. Details of these 29 isolates are in Table 1. An isolate (Pkce) from an unknown cultivar of sugarcane in the United States that was initially used to develop the SSR markers was included in the analysis as a positive

TABLE 1 Identification, cultivar of origin, year of collection and locality of *Puccinia kuehnii* from Brazil

Pathogen identification	Cultivar/ Lineage	Year/ Month	Locality
Pk02	SP891115	2014/Feb	Araras
Pk03	RB855511	2014/Jul	Araras
Pk04	RB855548	2014/Jul	Araras
Pk05	RB845197	2014/Jul	Araras
Pk06	RB995271	2014/Jul	Araras
Pk07	RB995152	2014/Jul	Araras
Pk08	404	2014/Jul	Araras
Pk28	Caiana	2015/Apr	Araras
Pk09	SP81-3250	2014/Oct	Pradópolis
Pk10	SP81-3250	2014/Oct	Pradópolis
Pk11	SP81-3250	2014/Jan	Orindiúva
Pk12	SP81-3250	2014/Feb	Sertãozinho
Pk13	SP81-3250	2014/ May	Sertãozinho
Pk14	SP81-3250	2014/Feb	Descalvado
Pk15	CTC20	2014/Feb	Descalvado
Pk16	CTC15	2014/Feb	Descalvado
Pk17	CTC15	2014/Jun	Descalvado
Pk18	SP84-2025	2014/Feb	Descalvado
Pk19	SP81-3250	2014/Feb	Descalvado
Pk20	SP81-3250	2014/Jun	Descalvado
Pk21	SP81-3250	2014/Feb	Descalvado
Pk22	SP81-3250	2014/Apr	Macatuba
Pk23	CTC15	2014/Sep	Leme
Pk26	RB055508	2014/Sep	Nova Europa
Pk27	RB05-5645	2014/Sep	Nova Europa
Pk25	SP81-3250	2014/Sep	Araras
Pk29	SP81-3250	2015/ Dec	Araras
Pk30	SP81-3250	2015/ Dec	Araras
ScPk01-01	SP81-3250	2018/Sep	Araras

control of SSR amplification. Nineteen primer sets that showed polymorphism in the initial screening of four isolates were chosen to screen the Brazilian isolates of *P. kuehnii*. The list of those 19 primers selected and their sequences is shown in Table 2.

The amplicon fragments in base pairs (bp) generated by running capillary electrophoresis for 19 SSR markers on 29 *P. kuehnii* isolates were converted to a binary matrix to estimate genotypic diversity of the Brazilian *P. kuehnii* isolates. Cluster analysis by neighbour-joining (NJ) (Nei, 1972) using Jaccard coefficient and 3D principal coordinate analysis (3D PCoA) were performed using software NTSYS 2.0 (F. James Rohlf, Exeter Software, Setauket, NY).

2.2 | Phenotypic diversity

2.2.1 | Host

The following sugarcane cultivars were used in the first screening experiment: RB835054 (72 young plants), RB867515 (308 plants), RB935744 (308 plants), RB966928 (72 plants) and RB975201 (308 plants). They were all classified as resistant to orange rust (Araújo et al., 2013, Porto & Urashima, 2018). In the subsequent experiment, cultivars RB935744 and SP81-3250 were employed, one as resistant and the latter as susceptible. Single-bud cuttings were produced from 9- to 11-month-old sugarcane stalks and planted in 770-ml plastic cups in a mixture (1 : 1 v/v) of soil and substrate (coconut fibre, vermiculite and pine-tree chips) amended with 3 g of 10-10-10 (NPK) fertilizer. Planted cuttings were maintained in a greenhouse for 30 days, at a temperature of $25 \pm 3^\circ\text{C}$, when they were inoculated. Only the soil was watered to keep the aerial part of the plants dry and prevent unwanted fungal infections.

2.2.2 | Inoculum preparation

Leaves of adult plants, around 9–12 months of age, of cultivars RB72454, SP81-3250 and SP89-1115 of sugarcane with suspected symptoms of orange rust were taken to the laboratory for identification of *P. kuehnii* as previously described. They were classified as highly susceptible and thus employed as orange rust spreader in the sugarcane breeding centre in Araras city, São Paulo state. Four- to six-leaf young plants were inoculated using the single-pustule method specifically developed for orange rust of sugarcane by Porto and Urashima (2018). Briefly, single pustules of type 6 lesion, according to the description of Tai et al. (1981), were selected and detached from the diseased leaves. This tissue piece containing urediniospores was rubbed over an area of 5 cm^2 of the abaxial part of a new sugarcane leaf, previously humidified with autoclaved distilled Milli-Q water and 0.01% of Tween 20; the urediniospore concentration in each inoculation was 1.10^5 spores per ml (Porto & Urashima, 2018). Negative controls were prepared on the distal area of the same leaves, towards the tip of the leaf by repeating the spread motion using a piece of healthy leaf over a 5 cm^2 area. Subsequently,

TABLE 2 List of simple-sequence repeat (SSR) markers designed for *Puccinia kuehnii*. Amplicons and number of alleles observed on 29 Brazilian isolates

ID	Orientation	Amplicons	Number of alleles
NPRL_PK_108	Forward: GAGAAATGCAATCCAAGAGGACAG	104–131	5
	Reverse: AAATGACGACATTGGAGACGTTG		
NPRL_PK_13	Forward: GATCCAGATCAACACCAGCCTC	177–419	3
	Reverse: ACTACCCATCATCATCCCATTGAG		
NPRL_PK_132_spka	Forward: GATATCATCGGGAACATCACTTTTG	151	1
	Reverse: AGGAAGGAGTCCCTATCAATCCTC		
NPRL_PK_135b	Forward: GAGGGAGCATCATATTCATCATCAC	177	1
	Reverse: CTTTCATCGACATCTTCATCACCAC		
NPRL_PK_16	Forward: GAGGAGATCAGGCTGCTTATCG	167	1
	Reverse: ACGTCACCAGAAGGAATAAGATCG		
NPRL_PK_162_spka	Forward: ACTTGTGAGTTTGACTTGCTGGG	124–180	4
	Reverse: CACCCATCTTCGTTCCAAGTAGTG		
NPRL_PK_19	Forward: ATCACTGCCTAATCCAAATTCAC	140–248	4
	Reverse: AGAGGAAAAGTGAAGAAGGCAAC		
NPRL_PK_20	Forward: AGCTGCCAATAAACACTCTTCTGG	140	1
	Reverse: AGAAGGAAGGGTCTGAAGAAGGAG		
NPRL_PK_221_spka	Forward: TTTCTGCTGACCGTTCATATCTTG	177–179	2
	Reverse: TGTAGAAGAGCAGGAAGAAGAGGG		
NPRL_PK_28	Forward: TGATCCATTTCAACTGTCAATGC	126	1
	Reverse: AGAAGAAGGAAGAGAGGTGGAAGC		
NPRL_PK_28_spka	Forward: GAGATGATCACCATGGCATTCTTAG	116	1
	Reverse: CTCCGCAACTAAAACCCTACAAAC		
NPRL_PK_37	Forward: CTCTAGCCTGTTCTTACTTGCTG	141–146	2
	Reverse: GAAACAATATTTGAACACTTTGACTCC		
NPRL_PK_382_spka	Forward: GAAGGTTCCGATTGAATTTTATTTCC	107–171	2
	Reverse: TAACTCTTCGTTCTTCTTTCCCC		
NPRL_PK_39	Forward: CGGTTACGACGAGAATACATTGC	108–406	10
	Reverse: GAGCATTCGAATCGTAAGACGAAG		
NPRL_PK_422_spka	Forward: ACCACTCAGGAAGACAGGAATGAG	104–113	2
	Reverse: TAGCCCTTCTTGCTGACTTGATTC		
NPRL_PK_49_spka	Forward: GTAACGGTGGTGTGCGTAACCTAG	181	1
	Reverse: ACGTAGCCCTCTTGCGTATAACC		
NPRL_PK_59	Forward: ATTTATTGTTGCAGAAAGTCAGGG	160–170	2
	Reverse: ATTAGGCGTATCCAACAACAATTC		
NPRL_PK_60_spka	Forward: ATAGCAGGCTGTTGAAGATTCAGG	261	1
	Reverse: TCTGAGAGACCCACAAACAAAATG		
NPRL_PK_89_spka	Forward: CAAGTATGGTCGTCCATCTCAACC	166	1
	Reverse: TGATTGATGATTGTGGAAGGATTG		

the whole plant was sprayed with distilled water, sealed in a plastic bag and kept in the dark at 25°C for 48 hr. After this infection period, plants were moved to a greenhouse with natural photoperiod and humidity, were protected from the rain and only the soil was watered to avoid secondary infections. Viability of urediniospores was examined at the time of inoculation by placing spore suspensions, at concentration of 10^5 spores per ml, on water-agar medium (4%)

in a Petri dish at 25°C for 10 hr. Subsequently, formation of germ tubes was verified under light microscope for 100 urediniospores in each of the four replications. In the initial screening assay, the same scheme was performed with SP89-1115 and SP81-3250 as source of pustules of orange rust for inoculation: 142 young plants of each cultivars RB935744, RB867515 and RB975201 and 24 young plants of each cultivars RB835054 and RB966928 were inoculated utilizing

one pustule on one plant, totalling 474 pustules of each, SP89-1115 and SP81-3250. When RB72454 was used as source of *P. kuenii*, 24 pustules inoculated each of above-mentioned cultivars, totalling 120 pustules of RB72454. In short, the initial screening assay employed 1,068 *P. kuenii* isolates on 1,068 young plants of five resistant sugarcane cultivars.

After the initial screening where cultivars and fungal isolates of interest were identified, a subsequent experiment was conducted in a repeated randomized block design experiment with five replications and one young plant per replication. This assay employed cultivars RB935744 and SP81-3250 and the single-pustule inoculation of two different sources of the *P. kuenii*: one from a wild isolate that caused orange rust symptoms on SP81-3250 in nature (PkSp01-01) and the other, the most virulent isolate of the initial screening assay isolate (PkSp01-01a4), which resulted in 19.63% of diseased area on RB935744 (Table 4). All inoculation procedures were as described for the initial screening as well as the pathogen concentration (10^5 spores per ml).

2.2.3 | Host reaction

Reaction of sugarcane cultivars to different isolates of *P. kuenii* was determined by measuring the size of rust-affected leaf area using Assess 2.0 software (APS Press, American Phytopathological Society, St. Paul, MN, USA). Measurements were taken 21 days post-inoculation using a leaf segment of 5 cm², each inoculation was considered one sample. Host/pathogen interactions were considered compatible, that is a virulent fungus on a susceptible host, when lesion areas were 1% or more of the inoculated area (5 cm²). On the other hand, when the diseased area was <1%, the interaction was considered incompatible. This threshold for classification in compatible/incompatible interaction has been adopted by all sugarcane breeding programmes in Brazil to classify breeding lines (Coelho et al., 1990; Chapola et al., 2016; Dinardo Miranda et al., 1998).

2.3 | Data analyses

Test of significance of phenotypic diversity was applied only on the duplicated assay. Data of percentage of disease were transformed to Log₁₀ ($x + 1$), if necessary to achieve normality and homoscedasticity, and transformed data were used for analysis of variance (ANOVA). Mean values were compared by the method of Tukey's HSD (Tukey, 1949), Scott-Knott or Kruskal-Wallis one way analysis of variance of ranks (Kruskal and Wallis, 1952). All analyses were performed using the statistical package SigmaPlot v. 12.5 (Systat Software Inc., San Jose, CA).

3 | RESULTS

All 19 SSR markers designed for *P. kuenii* resulted in amplifications, 10 were polymorphic, with number of alleles varying from two (five

markers: NPRL_PK_221_spka, NPRL_PK_37^a, NPRL_PK_382_spka, NPRL_PK_422_spka and NPRL_PK_59^a), three (NPRL_PK_13a), four (NPRL_PK_162_spka, NPRL_PK_19a), five (NPRL_PK_108a) and to 10 (NPRL_PK_39a). Nine SSR markers were monomorphic. SSR marker sequence, amplicon sizes and number of alleles detected by each marker are in Table 2. For further analyses, marker NPRL_PK_39a, which presented numerous null alleles, was not included in the cluster analyses. Genetic distances of 29 *P. kuenii* isolates showed four main clusters of isolates, as detected by NJ and 3D PCoA (Figures 1 and 2, respectively). The first cluster was formed by five isolates (Pk2, Pk4, Pk5, Pk6 and Pk7) retrieved from five different cultivars, but all from a sugarcane breeding site in a 4-month interval in 2014 (blue symbol) (Figure 1). The second one, composed by eight isolates (Pk11, Pk14, Pk17, Pk18, Pk19, Pk21, Pk22 and Pkce), included *P. kuenii* from three different cultivars but collected in the same location in Descalvado in 2014 as well as one isolate, Pk11 from Orindiúva, distant up to 320 km, and one (Pk22) from Macatuba forming the third apex, 170 km from Descalvado and 353 km from Orindiúva; the control sample of unknown origin was also placed in this group (Pkce) (pink). The third cluster included six isolates (Pk09, Pk10, Pk12, Pk13, Pk26 and Pk27) (green) collected from the same growing season (2014), cultivar (SP81-3250), and geographically separated around 40 km, two breeding lines (Pk26 and Pk27) distant 130 km from the others were also in the group. The fourth cluster (yellow) was the largest one with eight isolates and the only one with genotypic clones (Pk03, Pk15, Pk20, Pk23, Pk25, Pk28, Pk29 and Pk30) from three commercial cultivars (SP81-3250, CTC15 and CTC20), one breeding line (RB855511), one ancient *S. officinarum* cultivar (Caiana), and four different locations (two different growing sites in Araras city), and two crop seasons. Interestingly, isolates of *P. kuenii* from SP81-3250 were detected in three different clusters: pink (Pk19, Pk22), green (Pk13) and yellow (Pk20), demonstrating that one single cultivar can harbour genetically distinct isolates (Figures 1 and 2).

Alleles identified by amplification of three SSR markers, NPRL_PK_108a, NPRL_PK_162_spka and NPRL_PK_221_spka, discriminated the four clusters of isolates. They were named genotypes 1–4, for which the specific alleles of each genotype are listed in Table 3. Isolates Pk08 and Pk16 were not listed within the four groups since they represented intermediate genotypes.

Because viability of urediniospores of *P. kuenii* was above 50%, phenotypic diversity was examined on orange rust pathogens collected from three highly susceptible cultivars. The strategy adopted in this study, that is single-pustule inoculation on resistant cultivars allowed identification of races. When resistant cultivars RB835054, RB867515, RB935744, RB966928 and RB975201 were challenged by *P. kuenii* urediniospores inoculated by single-pustule technique, the great majority of isolates showed an incompatible reaction, as expected for resistant cultivars (Table 4). Nevertheless, a small proportion of pustules had urediniospores capable of causing orange rust symptoms on resistant cultivars. In detail, 1 out of 142 pustules (0.7%) of orange rust collected from cultivar SP89-1115 and inoculated on resistant cultivar RB935744 provoked a diseased area of

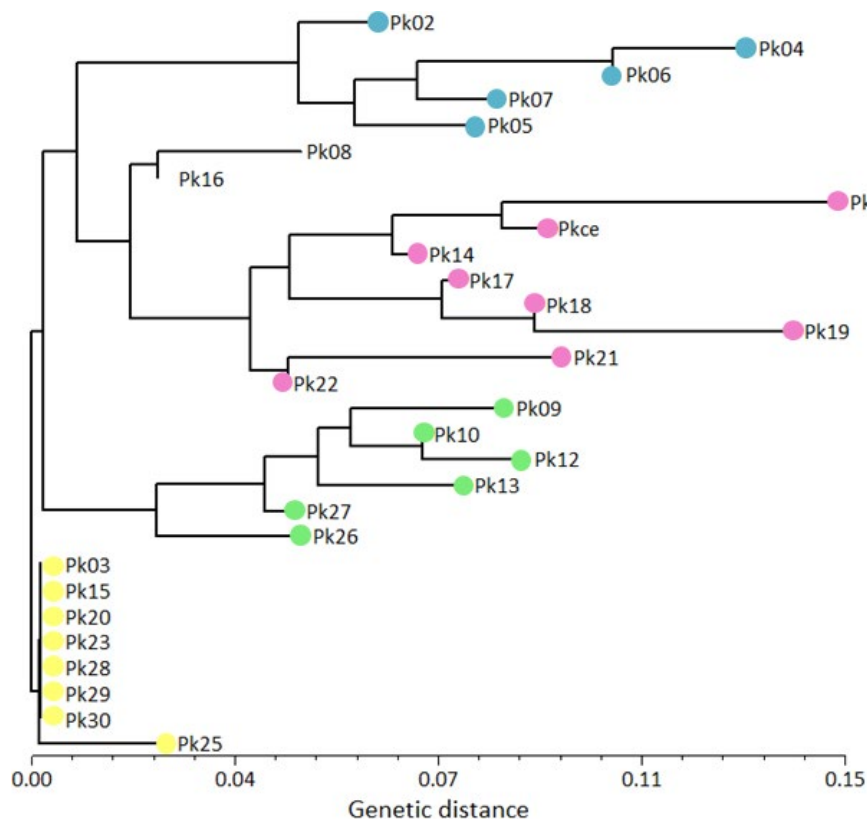


FIGURE 1 Four main groups of *Puccinia kuehnii* isolates clustered by neighbour-joining analysis of 18 SSR markers on 29 isolates. Cluster 1 (blue): Pk2, Pk4, Pk5, Pk6 and Pk7; Cluster 2 (pink): Pk11, Pk14, Pk17, Pk18, Pk19, Pk21 and Pk22; Cluster 3 (green): Pk9, Pk10, Pk12, Pk13, Pk26 and Pk27; Cluster 4 (yellow): Pk3, Pk15, Pk20, Pk23, Pk25, Pk28, Pk29, and Pk30

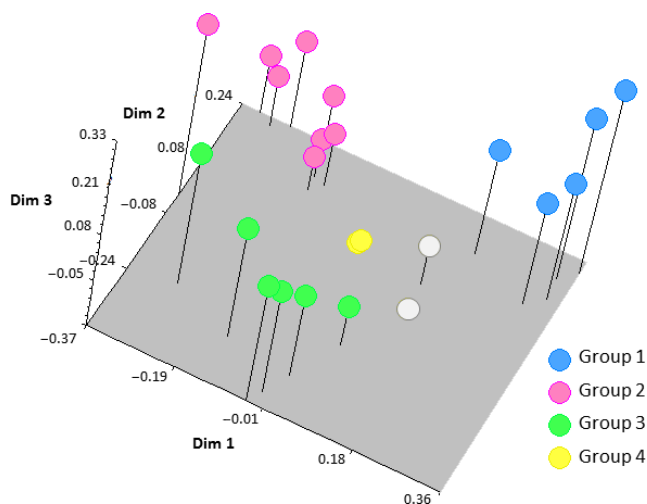


FIGURE 2 Four main groups of *Puccinia kuehnii* isolates clustered by 3D principal coordinate analysis of 18 SSR markers on 29 isolates. Cluster 1 (blue), Cluster 2 (pink), Cluster 3 (green) and Cluster 4 (yellow). Two isolates with intermediate genotypes are coloured grey

8.13%. Compatible reaction was also found in 3 out of 142 pustules (2.11%) in inoculation on resistant cultivar RB867515, diseased area varying from 13.69% to 4.98%; in 3 out of 24 pustules (12.5%) on RB835054, lesion area from 5.81% to 2.16%; in 1 out of 24 pustules (4.17%) on RB966928, lesion area of 5.03%; and in 1 out of 142 pustules (0.7%) on RB975201, lesion area of 7.13%. Altogether, susceptible cultivar SP89-1115 had 1.9% of the 474 pustules capable

of infecting orange rust symptoms on one of the five Brazilian resistant cultivars. Virulent sub-population was also found among 474 pustules collected from the susceptible cultivar SP81-3250, 1 out of 142 pustules showed compatible interaction on resistant cultivar RB935744, diseased area of 19.63%; 2 out of 142 pustules in each of the resistant cultivar RB867515 and RB975201, lesion size varying from 4.23% to 9.3%. Noteworthy was one pustule from SP81-3250 that caused a lesion of 19.63% on resistant cultivar RB935744, the greatest diseased area of all assay. In total, SP81-3250 harboured 1.05% of pustules capable of inciting orange rust symptoms on three sugarcane resistant cultivars. No virulent sub-population capable of infecting resistant cultivars was identified among 120 pustules of *P. kuehnii* retrieved from RB72454. Interestingly, the susceptible cultivar SP89-1115 seems to harbour the highest percentage of virulent sub-populations capable of infecting broadest number of resistant cultivars (five) as well as because despite the low number of pustule inoculated on resistant cultivars, 3 out of 24 pustules on RB835054 showed infecting ability and 1 out of 24 pustule caused compatible reaction on RB966928.

After the initial screening, a duplicate trial was conducted comparing field isolate from SP81-3250, named PkSp01-01, and the one retrieved from diseased area caused on resistant cultivar RB935744 (lesion area of 19.63%) and identified as PkSp01-01a4 (Figure 3). This figure showed results of two repeated experiments combined allowing a visual comparison of host reaction caused by these two fungal isolates. A clear distinction in virulence pattern was observed. The isolate PkSp01-01a4 caused lesion area varying from 6.08 to 3.31%, mean of diseased area of 4.88%, demonstrating a compatible

TABLE 3 Allele size amplified by three microsatellite markers among 29 isolates of *Puccinia kuehnii* that composed the four main genotypes identified

Marker	Genotype 1	Genotype 2	Genotype 3	Genotype 4
NPRL_PK_108 ^a	110	110	110 + 131 + 114 [*]	110
NPRL_PK_162_spka	140	124 (+some 153, 180)	140	140
NPRL_PK_221_spka	177	179	179	179
Isolates within each group	Pk02, Pk04, Pk05, Pk06, Pk07	Pk11, Pk14, Pk17, Pk18, Pk19, Pk21, Pk22	Pk09, Pk10, Pk12, Pk13, Pk26, Pk27	Pk03, Pk15, Pk20, Pk23, Pk25, Pk28, Pk29, Pk30

^{*}The discriminating alleles for each genotype are listed in bold.

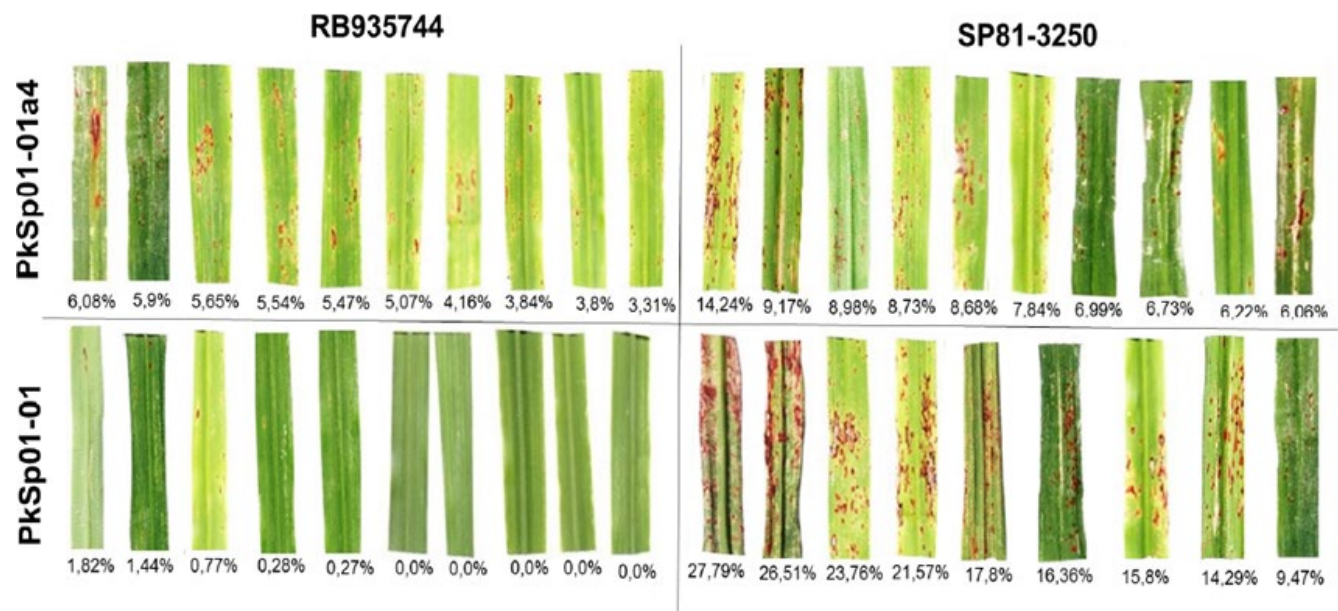
TABLE 4 Number of compatible/incompatible interaction and percentage of lesion area between five sugarcane resistant cultivars and *Puccinia kuehnii* by single-pustule inoculation

Inoculum source	Sugarcane resistant cultivars										Total of inoculations
	RB935744		RB867515		RB835054		RB966928		RB975201		
	(+) ^a	(-) ^b	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	
SP89-1115	1 (8.13) ^c	141	3 (13.69, 6.48, 4.98)	139	3 (5.81, 3.25, 2.16)	21	1 (5.03)	23	1 (7.13)	141	474
SP81-3250	1 (19.63)	141	2 (4.23, 6.64)	140	0	24	0	24	2 (4.85, 9.30)	140	474
RB72454	0	24	0	24	0	24	0	24	0	24	120
Total	2	306	5	303	3	69	1	71	3	305	1,068

^aCompatible interaction (1% or more of lesion area over an inoculated area of 5 cm²).

^bIncompatible interaction (lesion area below 1%).

^cNumber in parenthesis is the percentage of diseased area caused by each fungal isolate.

**FIGURE 3** Virulence of two different isolates of *Puccinia kuehnii* on two cultivars of sugarcane with differential reaction carried out in a repeated randomized block design experiment with five replications and one young plant per replication. Isolate PkSp01-01a4 was retrieved from single-pustule inoculation on a resistant cultivar (RB935744), and isolate PkSp01-01 was collected from a susceptible cultivar (SP81-3250)

interaction with RB935744. On the other hand, the wild isolate from SP81-3250 (PkSp01-01) caused lesion area from 1.82% to 0, mean of 0.46%, below the 1% threshold indicating an incompatible interaction. Moreover, the difference in virulence pattern was statistically

significant by Tukey's test; data of both assays were analysed combined because no interaction between assays existed resulting in increase of degree of freedom in the analysis (data not shown). Therefore, the single-pustule inoculation of these two isolates

confirmed existence of distinct fungal races of *P. kuehnii*. Both isolates had a compatible interaction with the original host SP81-3250, Scpk01-01a4 caused an average of 8.36%, varying between 6.60% to 14.24%, of lesion area whereas ScPk01-01 showed an average of 17.33%, with lesion varying from 9.47% to 27.79%.

4 | DISCUSSION

Data of the present work demonstrated that *P. kuehnii* infecting Brazilian sugarcane is genetically diverse, both at genotypic level and at phenotypic level. This finding was obtained by two independent studies since isolates employed at the genotypic assay were not the same from the phenotypic experiment. Therefore, any link between genotypic diversity and pathogen race was not possible in the present work. The molecular diversity was identified by SSR markers described in this work. They were developed from genome sequence of *P. kuehnii* collected from an American sugarcane cultivar (Tehen et al., 2010) and were useful for Brazilian rust pathogens from various geographic locations and cultivars, and, thus, have potential application for *P. kuehnii* of other sugarcane producing countries where orange rust disease is present. The usefulness of these markers was demonstrated since fragments generated by these primers in PCR amplification were clearly visible and of the expected size.

The diversity of the rust pathogens showed four distinct genotypic populations among the 29 of *P. kuehnii* isolates submitted to SSR analyses suggesting that even larger variability can be found in the most important sugarcane producing state should the number of rust isolates increases. This is best exemplified by SP81-3250, a source of *P. kuehnii* isolates collected from seven locations of six different municipalities of São Paulo state, which isolates grouped in three out of four genotypic groups (Figures 1 and 2). The diversity shown by isolates from this cultivar suggests that a clearer picture of genotypic diversity of *P. kuehnii* can be obtained by a broader sampling of orange rust on SP81-3250, a cultivar that still covers large areas in the central southern Brazil (Braga et al., 2018). The other two (RB72454 and SP81-3250) were already discarded by the Brazilian sugarcane industry. Therefore, there is no possibility of examining further diversity of *P. kuehnii* sampling other cultivars, but SP81-3250.

The great genotypic diversity detected in our work revealed two clusters (2 and 3) not present in a sugarcane breeding site, the location where new clones (or breeding lines) are challenged against biotic/abiotic stresses and then selected. Although conclusions may have been a little too hasty due to the limited number of isolates, the absence of sub-populations of *P. kuehnii* where selection of sugarcane genotypes is carried out leaves the possibility for a cryptic error in the breeding process (Correa-Victoria & Zeigler, 1993). This type of error may result in selection of germplasms that were not challenged against all variants of the pathogen population. This is a pivotal aspect in the selection process since a pathogen with great diversity has higher probability of rapid adaptation to the host and/or environment (Prasad et al., 2017). In this sense, one important step of breeding programmes to avoid cryptic error is increase the number of susceptible

cultivars employed as spreader of *P. kuehnii* during selection of breeding lines, usually only one is employed (Chapola et al., 2016).

The identification of three SSR markers capable of identifying the four genetic clusters of *P. kuehnii* was another important contribution of this work. The use of only three SSR markers to reveal the whole diversity of *P. kuehnii* improves strategies of orange rust control. It might increase the lifespan of new resistant cultivar by guiding breeding programmes to challenge genotypes against all pathogen variants during the selection process. Another asset of these SSR markers relates to monitoring and/or identifying new sub-population that may arise due to the dynamic of the host-pathogen interaction as observed in Egypt, where new races of *P. striiformis* f. sp. *tritici* were identified because of the distinct genetic clustering pattern compared to the one produced by the existing pathogen races (Draz, 2019).

Pathotypic diversity was also detected in our work by the existence of different races of *P. kuehnii*, where a significant interaction of two rust isolates on cultivar RB935744 was observed (Figure 3). The existence of races was first suspected on *P. kuehnii* on SP81-3250 because it was not mentioned as susceptible by the time of the first outbreak in 2009 (Barbasso et al., 2010), but subsequently showed severe epidemics in 2012–2013 (Raizen, 2013). Changes in cultivar reaction where canes with no foliar lesions displayed severe symptoms sometime after its release in fields were reported previously on sugarcane rusts in the United States, both for the brown rust on CP 70-1133, CP 72-1210 and CP 78-1247, and the orange rust on CP 88-1762 (Comstock et al., 2010). Indeed, the first documented outbreak of orange rust, on Q124 in Australia, was attributed to the same phenomenon (Braithwaite et al., 2009). Nevertheless, existence of fungal races is difficult to detect by field observations since temperature and leaf wetness among other factors, can increase aggressiveness, and consequently, disease severity (Barrera et al., 2013; Millus et al., 2009). Our data showed that the change in cultivar reaction from resistant to susceptible was due to the existence of races in *P. kuehnii*.

Races were identified in our work by the inoculation method employed. The single-pustule inoculation technique has previously proved a powerful tool in the case of UG99 of *P. graminis* f.sp. *tritici*. UG99 was a new race that emerged in Uganda, Africa, in 1999 with virulence to resistance gene *Sr31*, widely employed source of resistance to stem rust of wheat, reason why was considered a serious threat to wheat production worldwide (Pretorius et al., 2000). The race UG99 was then controlled by the resistant gene *Sr24* (Jin & Singh, 2006). Later however, the single-pustule inoculation revealed that UG99 actually harboured another race, virulent to the gene *Sr24* (Jin et al., 2008). This is an illustrating example of the strength of the single-pustule inoculation to examine the existing diversity of a pathogen, paramount for the success of disease control based on genotype resistance. Similarly, the single-pustule inoculation in the present work revealed that *P. kuehnii* infecting susceptible cultivars harboured a tiny sub-population capable of inflicting considerable symptoms on resistant cultivars.

The existence of fungal races is the main reason for breakdown of resistant cultivars in many host-pathogen interactions (Correa-Victoria & Zeigler, 1993; Prasad et al., 2017). Our phenotypic data proved that *P. kuehni* also features races, which is common in many

Puccinia species: *P. polysora* (Ryland & Storey, 1955), *P. striiformis* (Johnson et al., 1972) and *P. recondita* (Park et al., 2000), to name just a few. Moreover, our data showed that the virulent sub-population of *P. kuehnii* still corresponds to a tiny fraction of *P. kuehnii* present in susceptible cultivars and that a correct orange rust management control should be adopted to maintain this population in such level and thus minimize chances of future outbreaks of orange rust in Brazil. Conditions that favour orange rust symptoms include warm and humid conditions (Magarey et al., 2004), predominance of a single cultivar, covering large areas (Magarey, 2010), higher number of ratoon crops and inoculum pressure (Chapola et al., 2016), extended period of leaf wetness (Urashima et al., 2018) were already pinpointed. Nevertheless, one of the most important measures towards this strategy is the discard of susceptible cultivars, as recommended by the time of the first orange rust outbreak in Brazil (Barbasso et al., 2010). In sugarcane mills where this recommendation is followed, the urediniospore concentration of the virulent sub-population is likely to remain low and resistant cultivars, symptomless. Nevertheless, situations favouring increase of this sub-population can result in increase of urediniospores of the virulent sub-population resulting in disease symptoms on currently resistant cultivars. The first symptoms of orange rust were noticed when pathogen concentration reached 10^3 urediniospores per ml in a controlled inoculation assay (Urashima et al., 2018). One factor that favours augment of this sub-population is the widespread cultivation of susceptible cultivars. In this sense, a recent survey on the central south region, responsible for more than 90% of the Brazilian sugarcane production, revealed that five out of 10 most cultivated cultivars in the central southern Brazil during 2017–2018 crop season were susceptible to orange rust, namely RB855156, RB92579, SP83-2847, CTC15 and SP81-3250, representing 26.9% of 6.3 million hectares (Braga et al., 2018; Chapola et al., 2016). Of the most concern is SP81-3250 due to its popularity, been the fourth most cultivated in the 2017–2018 growing season with 5.8% (Braga et al., 2018), and a record of previous orange rust epidemics in 2012–2013 (Raizen, 2013). Therefore, it is pivotal for the industry to discard this cultivar despite its popularity; possibility exists of severe symptoms of orange rust on resistant cultivars such as RB867515, RB935744 and RB966928, due to the new fungal race present in small proportion.

Brazil is the largest sugarcane producer in the world and control of orange rust is an essential part of the industry. The outbreaks of this disease on popular cultivars revealed knowledge of pathogen diversity is crucial for the orange rust control based on resistant cultivars. The set of SSR markers developed and validated in this work revealed genotypic diversity even in a limited number of pathogen isolates. Existence of different fungal races was also disclosed here. Our data showed the need to implement measures to improve control of orange rust by resistant cultivars in Brazil: to breeding programmes, the importance of challenging breeding lines to the whole diversity of *P. kuehnii*, and to sugar mills, the urgent need to discard susceptible cultivars.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

PEER REVIEW

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