PATRÍCIA DA SILVA MACHADO

EPITYPIFICATION, CYTOLOGY, GENETIC AND PHYSIOLOGICAL VARIABILITY, AND GENOMIC ANALYSIS OF *Puccinia psidii*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae.*

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APROVADA: 26 de fevereiro de 2015.

Morag Glen

Poliane Alfenas Zerbini

Eveline Teixeira Caixeta

Sérgio Hermínio Brommonschenkel

Acelino Couto Alfenas
(Orientador)

To my parents who gave me the opportunity to go so far,
I dedicate this thesis

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BIOGRAFY

PATRÍCIA DA SILVA MACHADO, daughter of Baltazar Machado and Ilza da Silva Machado, was borned in February 13 of 1985 in Formiga, Minas Gerais, Brazil. She concluded her undergraduate in Forest Engineering in 2009 by the Universidade Federal de Viçosa, Viçosa, Minas Gerais. Master (2011) and Doctor Science (2015) in Plant Pathology at same institution with doctorate sandwich program realized at University of Tasmania, Australia. Since 2005 her academic activities has been under the guidance of Professor Acelino Couto Alfenas at the Forest Pathology Laboratory/ Bioagro.

SUMMARY

RESUMO

MACHADO, Patrícia da Silva, D.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Epitipificação, citologia, diversidade genética e fisiológica e análise genômica de *Puccinia psidii*.** Orientador: Acelino Couto Alfenas. Coorientador: Olinto Liparini Pereira.

Designou-se, neste trabalho, o epítipo de *Puccinia psidii* Winter, originário de *Psidium guajava* para servir como referência para futuros estudos taxonômicos e moleculares do fungo. Esta ferrugem é considerada autoécia e macrocíclica, com a fase de pícnio desconhecida e com poucas informações sobre a formação, condição nuclear e o papel dos basidiósporos no ciclo vital de *P. psidii*. Assim, a partir de estudos citológicos, encontrou-se que após a fusão nuclear e migração do núcleo diplóide do teliósporo para o metabasídio ocorre meiose e originam-se quatro núcleos haplóides. Formam-se septos entre cada um dos quatro núcleos, os quais podem sofrer divisão mitótica e resultar em quatro basidiósporos predominantemente binucleados. A partir de inoculações de basidiósporos em folhas destacadas de *Syzygium jambos*, observaram-se apressórios lobados rudimentares, mas sem evidência de penetração e infecção. Em outro estudo, seis marcadores microssatélites foram utilizados para analisar a variabilidade genética de 79 isolados de *P. psidii* coletados em diferentes hospedeiros da Austrália, Nova Caledônia e China. Até o presente momento os isolados são geneticamente uniformes, exceto para algumas mutações pontuais em quatro isolados da Austrália. Ainda que a correlação seja pequena ($p < 0.01$, $r = 0.061$), encontrou-se associação entre variabilidade genética de isolados de *P. psidii* do Brasil, estimada por microssatélites, e os resultados de inoculação cruzadas em diferentes espécies de mirtáceas. Realizou-se o sequenciamento completo e uma breve análise genômica de cinco isolados *P. psidii* do

Brasil e da Austrália. A montagem de cada isolado foi comparada com o isolado VIC42496. Mais de 70% dos contigs de cada isolado que não estavam presente na montagem do isolado VIC 42496 não exibiram homologia com nenhuma sequência depositada no banco de dados do NCBI. Para aquelas sequências que apresentaram similaridade com outras sequências fúngicas, incluindo *P. psidii*, apenas três contigs foram similares com genes que codificam produtos envolvidos em patogenicidade.

ABSTRACT

MACHADO, Patrícia da Silva, D.Sc., Universidade Federal de Viçosa, February, 2015. **Epitypification, cytology, genetic and physiological variability, and genomic analysis of *Puccinia psidii*.** Adviser: Acelino Couto Alfenas. Co-adviser: Olinto Liparini Pereira.

In this study, the epitype of *Puccinia psidii* Winter from *Psidium guajava* was designed to serve as reference for future taxonomic and molecular studies of the fungus. This rust is it considered autoecious and macrocyclic, but lacking pycnium and with little knowledge about the formation, nuclear condition and the role of basidiospores in the life cycle of *P. psidii*. Thus, cytological studies showed that after nuclear fusion the diploid nucleus migrated into the metabasidium and underwent meiotic division resulting in four haploid nuclei. Septum was formed between each nucleus, which can undergo mitotic division and result in four basidiospores mainly binucleate. After inoculations on detached leaves of *Syzygium jambos*, rudimentary lobate apressoria were observed, but with no evidence of penetration and infection. In another study, six microsatellite markers were used to analyze the genetic variability of 79 isolates of *P. psidii* collected from different hosts in Australia, New Caledonia and China. Up to now it was found that isolates are genetically uniform, except for a few point mutations in four isolates from Australia. Although the correlation is low ($p < 0.01$, $r = 0.061$), there was an association between the genetic variability of isolates of *P. psidii* from Brazil, estimated by microsatellites, and the results of cross-inoculation on different Myrtaceae species. Genome sequencing of five isolates of *Puccinia psidii* from Brazil and Australia and a brief genomic analysis among them was provided. The assembly of each isolate was compared to VIC 42496. The analysis reveled that more than 70% of the contigs of

each isolate that was not present on assembly of VIC42496 had no significant homology (no hits) to anything currently residing in the fungal genome databases. For those contigs that displayed similar to fungal sequences, including *P. psidii*, only three contigs matched with genes coding for products that could be involved in pathogenicity.

GENERAL INTRODUCTION

Puccinia psidii, was originally described in 1884 in plants of *Psidium guajava* (= *Psidium pomiferum* L.) in São Francisco do Sul/SC, Brazil (Winter, 1884). The pathogen has been reported on about 56 genera and 244 species of Myrtaceae. Due to its broad host range, mass urediniospore production and its high capacity to be dispersed over long distances, the disease is a global threat to biodiversity of native species and exotic commercial crops (Loop & La Rosa 2008, Tommerup et al. 2003, Uchida et al. 2006, Glen et al. 2007). Despite the importance of *P. psidii*, a holotype was not designated by Winter and due to the obligately biotrophic nature of the pathogen and the age of the original collection, efforts to amplify DNA extracted from this specimen have been unsuccessful (Glen unpublished). The absence of reference for the species has led to some taxonomic confusion (Simpson et al. 2006, Carnegie et al. 2010).

Basidium, aecium, telium and uredinium stages of the pathogen are produced in the same host (autoecious) and aeciospores and urediniospores are morphologically identical. Basidiospores of *P. psidii*, supposedly uninucleate, can infect *S. jambos* (Figueiredo 2001). However, as the fungus does not produce spermogonia is not clear how dicaryotization takes places in *P. psidii* lifecycle. Other events are still unclear such as formation of metabasidium and basidiospores, number of nuclei, karyogamy, meiosis and other nuclear events that are sources of genetic variability, essential for the emergence of new races.

In April 2010, *P. psidii* was reported on *Agonis flexuosa* in Australia (Carneguie et al. 2010), the centre of origin of eucalypt, melaleuca, and several myrtaceous species. Artificial inoculations showed that at least 107 Australian native hosts in 30 genera are susceptible to the rust (Carnegie and Lidbetter 2012). In Brazil, the pathogen is considered endemic (Tommerup et al. 2003) and is not usually severe on native hosts with the exception of occasional epidemics in guava orchards (de Goes et al. 2004, Ribeiro and Pommer 2004), but the rust is an important pathogen to eucalyptus in nurseries and plantations (Alfenas et al., 2009). In the field, plants are more susceptible up to two years old and on coppice after clear-cutting. Infected plants rarely die, but due to necrosis and hypertrophy of twigs and branches there is loss of apical dominance and growth (Ferreira 1989, Coutinho et al. 1998).

Compared to other rust species, there are few studies to identify races of *P. psidii*. In a study realized by Xavier (2002) 3 races of *P. psidii* were identified, race 1 (isolated UFV2) is considered the most common in Brazil, while races 2 and 3 are rare and able to overcome, respectively, the resistance of the clones 1205 (*E. grandis*) and 847 (*E. urophylla*). These three races do not infect plants containing the Ppr-1 gene, a major effect gene that controls resistance to rust in a clone G21 of *E. grandis* (Junghans et al. 2003). However, a new race (4) of *P. psidii* was able to infect a *E. grandis* hybrid clone (6021) resistant to race 1. Under controlled inoculations, the new race was also able to infect a wide range of resistant clones to race 1 including clone G21 (*E. grandis*), carrying the resistance gene Ppr-1 (Graça et al. 2011).

Besides the *P. psidii* races infecting eucalyptus, cross inoculations of the pathogen on different species of Myrtaceae in Brazil have indicated for several decades the existence of different physiological groups of *P. psidii* with different, overlapping host ranges (Castro et al. 1983, Coutinho & Figueiredo 1984, Ferreira 1989, Coelho et al. 2001, Xavier 2002, Aparecido et al. 2003). A recent molecular study in Brazil using microsatellite markers has shown a likely pathogen selection according to host species regardless of geographic location (Graça et al. 2013). The existence of host-species genotypes may indicate the occurrence of cryptic species within the complex *P. psidii* or it is possible that the pathogen is co-evolving to a level of ecological specialization "formae speciales" or even to species level. However, there are no studies that combine cross inoculations with molecular techniques to prove this hypothesis. That hypothesis and other gaps of the knowledge of *P. psidii* can be clarify by the genome sequencing. This approach has provided insights into genetic changes related to the obligately biotrophic (Duplessis et al. 2009), difference between races or species (Duplessis et al. 2011), factors of pathogenicity and virulence (Cantu et al. 2011).

This study aimed: a) To characterize and describe an epitype of *P. psidii* from *Psidium guajava* to serve as reference for the pathogen; b) To evaluate the formation, nuclear condition and infectivity of basidiospores of *P. psidii*; c) To access the genetic variability of *P. psidii* population from Australia by microsatellite markers; d) To evaluate the association between genetic variability of *P. psidii* scored by microsatellite markers and results of cross-inoculations on different host species; e) To compare the genome sequencing of five isolates of *P. psidii*.

This thesis comprises the following five articles: 1) Epitypification of *Puccinia psidii*, causal agent of guava rust; 2) Formation, nuclear condition and infection of basidiospores of *Puccinia psidii*; 3) Microsatellite analysis of *Puccinia psidii* population in Australia; 4) Genetic and physiological variability of *P. psidii* population; and 5) Comparative genomic analysis of *Puccinia psidii* isolates.

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ARTICLE 1

Epitypification of *Puccinia psidii*, causal agent of guava rust

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ABSTRACT

Puccinia psidii is an important pathogen considered a threat to plants of the Myrtaceae around the world. Morphological variability has created misunderstanding around the classification of this pathogen. Due to the obligately biotrophic nature of *P. psidii*, the age of the material on which the original description was based, and the poor condition of its DNA, there has been no clear genetic characterization of the species. An epitype is therefore designated for *P. psidii* for precise application of this taxon name. A morphological description and DNA characterization are provided from the epitype, obtained from the same location and host of the lectotype, also designated herein. Morphologically, the epitype is identical to the original material and phylogenetic and microsatellite analyses show that the epitype is representative of *P. psidii* from guava in Brazil.

Key-words: Forest pathology, Myrtaceae, myrtle rust, plant pathogen, rust fungi, *Psidium guajava*, *Psidium pomiferum*.

Introduction

Puccinia psidii Winter, known colloquially as guava, eucalyptus or myrtle rust, was originally described in 1884 on guava, *Psidium guajava* L. (= *Psidium pomiferum* L.), from São Francisco do Sul, Santa Catarina, Brazil (Winter, 1884). Up to now the pathogen has been reported on about 56 genera and 244 species in the Myrtaceae in South America (Coutinho et al., 1998), Central America, Caribbean (Maclachlan, 1938; Laundon & Waterston, 1965), Florida (Marlatt & Kimbrough 1979), California (Mellano, 2006), Hawaii (Uchida et al., 2006), Japan (Kawanishi et al., 2009), Australia (Carnegie et al., 2010, Pegg et al., 2014), China (Zhuang & Wei, 2011) and more recently in South Africa (Roux et al., 2013) and New Caledonia (Giblin, 2013). Due to the broad host range, prolific urediniospore production and its capacity to be dispersed over long distances (Glen et al., 2007), the pathogen is a global threat to commercial crops such as *Eucalyptus* spp., *Psidium guajava*, *Pimenta dioica* (L.) Merr and *Melaleuca* spp. (Coutinho et al., 1998; Tommerup et al., 2003; Uchida et al., 2006; Loop & La Rosa 2008) and is especially threatening to native biodiversity where the native biome is dominated by Myrtaceae species, such as Hawaii and Australia (Uchida et al., 2006; Carnegie & Lidbetter, 2012).

The broad host range of *P. psidii* led to some taxonomic confusion as early investigators in South America described rusts on different myrtaceous hosts as new species, mainly in the genera *Puccinia* and *Uredo*, but also *Aecidium* Pers., *Caeoma* Link and *Bullaria* DC. Most of the species were subsequently synonymized with *P. psidii* (Arthur, 1922; Jackson 1931; Walker 1983; Simpson et al., 2006). Analyses of several collections of *P. psidii*, including the original specimen, found that most urediniospores are ellipsoidal to ovoid and completely echinulate (Simpson et al., 2006).

However strains collected on *Myrtus communis* L. in Argentina and *Syzygium jambos* (L.) Alston in Jamaica had ovoid to pyriform urediniospores with a distinct tonsure or bald spot (Walker, 1983). Simpson et al. (2006) classified these collections with tonsured spores as a new species, *Uredo rangelii* Simpson, with the specimen on *M. communis* designated as the holotype (DAR 31011). *Uredo rangelii* was subsequently reported in Australia in 2010 on *Agonis flexuosa* (Carnegie et al., 2010). With the discovery that *U. rangelii* produced teliospores, Carnegie and Cooper (2011) considered it a synonym of *P. psidii*.

The correct systematic position of plant pathogenic fungi is important for breeding programs, management of disease and formulation of trade and biosecurity policies (Cai et al., 2011). In the last decade there has been a revolution in the taxonomy of plant pathogenic fungi, with the application of molecular phylogenetic methods that reveal cryptic speciation (Shivas & Cai, 2012). Morphological and molecular data are reliable if obtained from type specimens. If type (holotype, isotype, lectotype or neotype) specimens are not available, an epitype may need to be designated (Hyde & Zhang, 2008). An epitype is “a specimen or an illustration selected to serve as an interpretative type when the holotype, lectotype, or previously designated neotype, or all original material associated with a validly published name, is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name of a taxon” (McNeil et al, 2012). Due to the obligately biotrophic nature of *P. psidii* and the age of the original collection, efforts to amplify DNA extracted from this specimen have been unsuccessful (Glen unpublished). Given the importance of the pathogen and the poor quality DNA of the original material, the aims of this study were to epitypify *P. psidii* and genetically characterize the epitype.

Materials and methods

Sampling

Several specimens of *P. psidii* were collected from *Psidium guajava* around São Francisco do Sul, Santa Catarina, the same host species and location from which the original specimen described by Winter was collected (Table 1). Symptomatic leaves and fruits were collected, photographed, and dried in a plant press. Representative specimens were deposited in the herbarium at the Universidade Federal de Viçosa (VIC), Orange Agricultural Institute (DAR), and Queensland Plant Pathology Herbarium, Australia (BRIP).

Other *P. psidii* samples collected from different hosts and locations in Brazil and elsewhere (Table 2) are from the fungus collections of the Forest Pathology Laboratory (Universidade Federal de Viçosa) or Plant Pathology Laboratory (University of Tasmania). The specimens were collected from the field, and individual pustules (containing fungal and host tissue) were stored at –80°C until DNA extraction.

Morphology

Teliospores and urediniospores collected from infected leaves and fruits were examined under light (Olympus BX53) and scanning electron microscopes (SEM). For light microscopy the samples were mounted in lactophenol, and for SEM, fragments (5 mm^2) of guava leaves containing uredinia and urediniospores were fixed in osmium tetroxide vapor for 12 h. Subsequently, the fragments were mounted on a metal support and metalized with a 150-Å layer of gold and examined using a LEO 1430VP scanning electron microscope (Zeiss, Cambridge, UK). Spores preserved in 70% ethanol were dehydrated in an ethanol series and dried at room temperature. Subsequently, the spores were mounted on a metal support and metalized with platinum in a sputter coater (Bal-Tec SCD 050) and examined using a Hitachi SU-70 field emission SEM.

DNA extraction

Whole genome amplification was attempted from genomic DNA extracted from the original 1884 material using the procedures of Glen et al. (2002). Three small pustules were excised, placed in a 1.5 mL microcentrifuge tube and ground with a disposable, plastic grinder after freezing with liquid nitrogen. A total of 250 µL extraction buffer (Raeder & Broda 1985) was added and the tubes incubated at 65°C for 1 h. Tubes were centrifuged at 14,000 rpm for 15 min and the supernatant removed. DNA was purified by binding to glassmilk. Briefly, 600 µL of 100% NaI and 10 µL glass milk (silica) was added to 200 µL of the supernatant and vortexed. The mixture was incubated on ice for 15 min with occasional shaking. Tubes were centrifuged for 10 s at 14,000 rpm, the supernatant removed, and the pellet resuspended in 600 µL of wash buffer (100 mM NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA in 50% ethanol). Following centrifugation for 10s at 14,000 rpm, the supernatant was removed, the pellet suspended in 600 µL 100% ethanol and centrifuged as before. Finally, the supernatant was removed and the pellet dried for 20 min. DNA was eluted by adding 20 µL of TE buffer, vortexing briefly and incubating at 45°C for 10 min. Supernatant containing DNA was removed following centrifugation for 2 min at 14,000 rpm and stored at –20°C. Whole genome amplification was attempted using a Repli-g minikit (Qiagen, Hilden, Germany), following the kit instructions.

Genomic DNA for PCR and microsatellite analyses was extracted from urediniospores collected on guava leaves in 2013 using a Wizard Genomic DNA Purification kit (Promega Corporation, WI, U.S.A.) with some adjustments as follows: urediniospores collected from the field and from inoculated plants were placed separately in a 2.0 mL microcentrifuge tube with two 3 mm tungsten carbide beads

(Qiagen, Hilden, Germany) and 200 µL Nuclei Lysis Solution (Promega Corporation, WI, U.S.A.), then macerated in a Tissuelyser II (Qiagen, Hilden, Germany) for 2 min at 30 Hz. The tubes were incubated at –20°C for 5 min, an additional 400µL Nuclei Lysis Solution was added and a second round of maceration performed. Tubes were incubated at 65°C for 15min, then centrifuged at 20,000 g for 7 min. The supernatant (about 400 µL) was transferred to a clean 1.5 mL tube and 300 µL Protein Precipitation Solution (Promega Corporation, WI, U.S.A.) was added. Tubes were centrifuged at 20,000 g for 10 min, then the supernatant (about 500 µL) was transferred to a clean 1.5 mL tube and phenol: chloroform: isoamyl alcohol (25:24:1) (Life technologies, Carlsbad, USA) was added. Tubes were centrifuged at 20,000 g for 5 min and the aqueous phase (approx. 400 µL), was transferred to a new microcentrifuge tube containing 600 µL isopropanol and 50 µL sodium acetate. Tubes were vortexed before an overnight incubation at -20°C. Tubes were centrifuged at 20,000 g for 7 min and the supernatant was discarded. Cold ethanol (600 µL) was added to the pellet and tubes centrifuged at 20,000 g for 5 min. The supernatant was discarded and the ethanol wash repeated, after which the supernatant was again discarded and the tubes inverted to dry the pellet. Finally, the pellet was re-suspended in 30 µL DNA Rehydration Solution (Promega Corporation, WI, U.S.A.) and 1 µL RNase Solution (Promega Corporation, WI, U.S.A.) and incubated at 37°C overnight. For the other *P. psidii* isolates (Table 2), genomic DNA was extracted directly from each pustule (containing fungal and host tissue) using a modified CTAB-based protocol (Graça et al., 2013).

PCR, sequencing and phylogenetic analysis

Three different regions were amplified by polymerase chain reaction (PCR): a portion of the β-tubulin gene using primer Ppsi-BtubF

(5'CTTTTGGTTCACTCTTCAGACC3') and Ppsi-BtubR (5'AGATGATAAAAGACTACTGACTCC3'), the internal transcribed spacer region of the ribosomal DNA (ITS) using primers ITS-1F (5'CTTGGTCATTTAGAGGAAGTAA3') (Gardes & Bruns, 1993) and ITS-Rust (5'GCTTACTGCCTTCCTCAATC3') (Kroop et al., 1995) and a portion of the transcription elongation factor EF1-α using primer PPEFF (5'AAGGATGCTGCTGACATGGGC3') and PPEFR (5'ATCCCGAAATGGGGACAAAAGG3'). Each 25 µL PCR reaction contained 5.0 µL of 5x Reaction Buffer (Bioline), 2mM $MgCl_2$ (Bioline), 0.2 mg/mL Bovine Serum Albumin (BSA, Fisher BioReagents™), 200 µM each dNTP, 1U of Taq DNA polymerase (Bioline), 0.25 µM of each primer (forward and reverse), 12.55 µL of water and 5 µL of genomic DNA. PCR amplifications were performed using a thermal cycler (model 2720, Applied Biosystems) and the following program for the primers Ppsi-BtubF/Ppsi-BtubR and PPEFF/PPEFR: 95ºC for 3 min, then 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, followed by 72°C for 7 min, ending with a hold at 14°C. For the ITS1F/ITSRust primers, the program was: 94ºC for 2 min, then 30 cycles of 94°C for 30s, 44°C for 30s, 72°C for 2 min, followed by 72°C for 10 min, ending with a hold at 14°C. The PCR products were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). Chromatograms were viewed and sequences edited using Chromas Lite version 2.1.1 software (Technelysium Pty Ltd).

Additional sequence data from samples of *P. psidii* collected from different hosts and locations in Brazil and elsewhere were included in the phylogenetic analysis (Table 2). DNA sequences were aligned using Clustal X (Larkin et al., 2007). The sequences of the three genes were concatenated and *Puccinia graminis* f. sp. *tritici* was used as the out-group. Additional sequences from *Puccinia* species with high similarity to *P. psidii* at individual loci were also included in the analysis (Table 2). As only single locus sequences were available for these four species, the other sequences were treated as missing data. A Bayesian analysis was performed using MrBayes v.3.2.1 (Huelsenbeck & Ronquist, 2001), using a GTR model with gamma-distributed rate variation, adding generations until the standard deviation of split frequencies was less than 0.01, discarding 25% of trees for burn-in. Sequences derived from this study were lodged in GenBank.

Microsatellite genotyping

The epitype specimen and 13 additional samples collected from guava in São Francisco do Sul and neighborhood regions were genotyped at six microsatellite loci (EF523503, EF523504, EF523507, EF523508, EF523510, EF523513) (Zhong et al., 2008; Graça et al., 2013). For each 10 µL PCR reaction we used 5 µL of 2x Master Mix (Type-It Microsatellite PCR kit, Qiagen), 0.1 µL (20 µM) of forward primer (labelled with either D2, D3 or D4 Well-RED fluorescent dye, Sigma-Aldrich) and reverse primer, 0.2 mg/mL of bovine serum albumin (BSA, Fisher BioReagents™), 3.6 µL of nuclease-free water and 1 µL genomic DNA. PCR amplifications were performed using a thermal cycler (model 2720, Applied Biosystems) and the following program: 95ºC for 3 min, then 34 cycles of 94°C for 15s, 45 to 50°C (depending on the locus) for 15s, 72°C for 45s, ending with 60 °C for 30 min, then 14°C. Fragment analysis was conducted on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter), using 1 µL of PCR product mixed with 38.5 µL Sample Loading Solution (Beckman Coulter) and 0.5 µL size marker (DNA Size Standard Kit – 400, Beckman Coulter).

Inoculation

To confirm the pathogenicity and to establish a strain to multiply for DNA isolation, urediniospores were inoculated on two susceptible cultivars (Pedro Sato and Paluma) of *Psidium guajava*. Grafted plants of both cultivars were maintained in 2 L plastic bags containing the substrate MecPlant® (pine bark partially decomposed and vermiculite), supplemented with 3 Kg/m³ of Osmocote® and 8 Kg/m³ of simple super phosphate. A string was used to mark young leaves that were inoculated. For inoculation, a soft bristle brush was used to spread the urediniospores on the leaves. After inoculation, plants were incubated for 24 h in a mist irrigation chamber at 25±2°C in the dark, and then they were transferred to a growth chamber at 22±2°C with a 12 h light cycle (80.µmol/m².s) (Ruiz et al., 1989). Approximately 12-20 days after inoculation, urediniospores were collected by brushing onto aluminium foil using an autoclaved, soft bristle brush, and stored at -80°C.

Results

According to the microsatellite results, the estimated alleles sizes (pb) for the loci EF523503, EF523504, EF523507, EF523508, EF523510, EF523513 are respectively: 217/219, 144/146, 160/160, 143/143, 237/237, 210/212. All samples collected from guava in São Francisco do Sul and neighborhood had the same microsatellite alleles, except VIC 42497, which differed at locus EF523508, with allele sizes of 139/139 rather than 143/143. Inoculations of spores of VIC 42496 on “Paluma” and “Pedro Sato” guava plants produced abundant urediniospores approximately 12 days after inoculation. The multilocus genotype of the spores collected from the inoculated plants was identical to VIC 42496.

All attempts to amplify DNA extracted from the original type material collected in 1884 and from the whole genome amplification were unsuccessful. The DNA sequences for the epitype and the other *P. psidii* samples from Brazil, Uruguay, Hawaii, Australia, and New Caledonia were identical, and the individual or combined analyses of the three different regions (β-tubulin, ITS, and transcription elongation factor EF1-α) demonstrated that the samples of *P. psidii* constituted a strongly supported monophyletic clade, distinct from the most closely related species for which sequences have been published (Figure 1). There was no amplification for the sample EGLU using the primers ITS1F/ITS RUST, nor for sample OAHU8 using EF1-α primers. Among all the samples collected in São Francisco do Sul and neighboring regions, VIC 42496 was selected as the epitype due to the presence of representative teliospores and urediniospores.

Taxonomy

Puccinia psidii G. Winter, Hedwigia. 23: 172. 1884.

(Figures 2-3)

Uredia abundant on the leaves, buds and fruits. Urediniospores echinulate, sometimes with a basal tonsure, hyaline to light-yellow, globose 18-23 µm diam. to ovoid, 20-26x 15-22 µm, wall 1.5-2.5 µm thick. Teliospores with a median septum, dark yellow to brown, ellipsoidal to ovoid, 27-43 x 16-24 µm, wall 0.7-1.0 µm, pedicel 9-13 µm long. Specimens examined: BRAZIL. São Francisco do Sul, on *Psidium guajava* (=*Psidium pomiferum* L.), Apr. 1884, E. Ule #14, BR-MYC 80409,93 (ex Museum Botanicum Berolinense) **to be proposed as lectotype**; DAR 29790 (microscope slide) **to be proposed as isolectotype**; Araquari, on *Psidium guajava*, 6 Feb. 2013, A.C. Alfenas & P.S. Machado, VIC 42496 **to be proposed as epitype**; BRIP 61521 **to be proposed as**

iso-epitypes; São Francisco do Sul, on *P. guajava*, 5 Feb. 2013, A.C. Alfenas & P.S. Machado, VIC 42489 to VIC42494; Araquari, on *P. guajava*, 6 Feb. 2013, A.C. Alfenas & P.S. Machado, VIC 42495, 42497, VIC 42499 to VIC 42503. ARGENTINA, Buenos Aires, on *Myrtus communis*, 12 Oct.1970, A. Burkart, DAR 31011.

Discussion

Amplification from DNA extracted from the type material of *P. psidii* collected in 1884 during a previous study (Langrell et al., 2008) and from the whole genome amplification of the same DNA sample were unsuccessful, probably because of the poor condition of the specimen. The collection designated as the epitype was collected from the same host and geographical area as the material from the original description, and the morphological characteristics of urediniospores and teliospores agreed with the original description (Winter, 1884). The microsatellite genotyping showed that the epitype VIC 42496 and another 13 samples collected from *P. guajava* in São Francisco do Sul and neighborhood have the same genotype, with one minor exception. According to a recent population study based on microsatellite markers, genotypes of *P. psidii* samples on guava at São Francisco do Sul are similar to those found on guava throughout Brazil (Graça et al., 2013). Guava plants inoculated with urediniospores from the epitype VIC 42496 produced abundant urediniospores, however, no teliospores were seen. The epitype and all *P. psidii* collections, including collections from Australia and New Caledonia, had identical DNA sequences for three genetic regions and constituted a strongly-supported monophyletic clade with no support for any further subdivision, though these regions are known to be polymorphic and informative for other rust genera, including *Chrysomyxa* (Feau et al., 2011), *Melampsora* (Vialle et al., 2013), *Phakopsora* (Chatasiri & Ono, 2008) and *Puccinia* (van der Merwe et al., 2008).

The current concept of *P. psidii* is based on morphological characters. While not mentioned in the original description of *P. psidii*, the presence of tonsures on urediniospores of *P. psidii* has been observed as a variable character (Walker, 1983; Simpson et al., 2006; Pérez et al. 2011), and DNA sequence analyses show a surprising level of uniformity. Problems in fungal identification due to morphological variability were also reported in other rust fungi (Berndt, 2011; Feau et al., 2009; Feau et al., 2011, Vialle et al., 2013). Ex-epitype isolates of *P. psidii* are being multiplied at Universidade Federal de Viçosa for future comparative genomic analysis.

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Table 1. List of *Puccinia psidii* samples, including epitype (in bold), collected on *Psidium guajava* in São Francisco do Sul, Araquari and Itajaí, Santa Catarina (SC).

VIC number	Location	GPS coordinates	
42489	São Francisco do Sul	S26.235583	W48.629389
42490	São Francisco do Sul	S26.243611	W48.634056
42491	São Francisco do Sul	S26.243611	W48.633889
42492	São Francisco do Sul	S26.243611	W48.634056
42493	São Francisco do Sul	S26.261056	W48.616472
42494	São Francisco do Sul	S26.262306	W48.616889
42495	Araquari	S26.442139	W48.642889
42496	**Araquari**	**S26.3735**	**W48.716278**
42497	Araquari	S26.372722	W48.723833
42499	Araquari	S26.372722	W48.723833
42500	Araquari	S26.372722	W48.723833
42501	Araquari	S26.371306	W48.69175
42502	Araquari	S26.370889	W48.711056
42503	Itajaí	S26.9083	W48.6626

Table 2. List of *Puccinia psidii* sequences used in the phylogenetic analysis, including epitype (in bold) and outgroup species for which sequences were obtained from GenBank.

Isolates	Host	Location	GenBank Accession Code			Reference
			β- tubulin	**ITS**	**EF1-α**	
EGUFV2	*Eucalyptus grandis*	Itapetininga / São Paulo/Brazil	KM282115	KM282148	KM282142	This Study
EUGBA1	*Eucalyptus urophylla* x *Eucalyptus grandis*	Teixeira de Freitas/ Bahia/Brazil	KM282127	KM282152	KM282141	This Study
EGLU	*Eucalyptus globulus*	Montevideo/ Uruguay	KM282110		KM282128	This Study
PGOP17	*Psidium guineense*	Conselheiro Lafaiate/ Minas Gerais /Brazil	KM282122	KM282149	KM282133	This Study
VIC42496	***Psidium guajava***	**Araquari/ Santa Catarina/Brazil**	**KM282123**	**KM282154**	**KM282143**	**This Study**
PGOP12	*Psidium guajava*	Lavras Novas/ Minas Gerais /Brazil	KM282117	KM282151	KM282145	This Study
PGES21	*Psidium guajava*	Venda Nova do Imigrante/Espírito Santo/Brazil	KM282116	KM282147	KM282144	This Study
EGUMG1	*Eugenia uniflora*	Viçosa/ Minas Gerais /Brazil	KM282119	KM282155	KM282135	This Study
EGUV	*Eugenia uniflora*	MG/Brazil	KM282124	KM282146	KM282134	This Study
MCMG1	*Myrciaria cauliflora*	Teixeiras/ Minas Gerais /Brazil	KM282118	KM282162	KM282136	This Study
MCSP10	*Myrciaria cauliflora*	Alambari/ São Paulo /Brazil	KM282120	KM282157	KM282137	This Study
SJSUZ14	*Syzygium jambos*	Raul Soares/ Minas Gerais/Brazil	KM282125	KM282161	KM282138	This Study
SJPS47	*Syzygium jambos*	Hawaii/United States	KM282111	KM282160	KM282130	This Study
SJSP9	*Syzygium jambos*	Alambari/ São Paulo/Brazil	KM282121	KM282150	KM282140	This Study
SJMG1	*Syzygium jambos*	Viçosa/ Minas Gerais/Brazil	KM282126	KM282158	KM282139	This Study
SJNC3	*Syzygium jambos*	New Caledonia	KM282112	KM282156	KM282131	This Study
BRIP59519a	*Melaleuca leucadendron*	Cairns/ Queensland/Australia	KM282113	KM282159	KM282132	This Study
OAHU8	*Syzygium jambos*	Oahu/ Hawaii/ United States	KM282114	KM282153	KM282129	This Study
Puccinia poae-nemoralis	*Anthoxanthum odoratum*		EF570827.1			Van der Merwe et al.,2008
Puccinia geranii-pilosi	*Geranium* sp.		EF570852.1			Van der Merwe et al.,2008
Puccinia saccardoi	*Goodenia hederacea* subsp. *alpestris*				DQ925285.1	Van der Merwe et al.,2007
Puccinia fergussonii	*Viola palustris*				DQ925264.1	Van der Merwe et al.,2007
Puccinia graminis f. sp. *tritici*			XM_003330619.2	XM_003330856.2	XM_003333024.2	

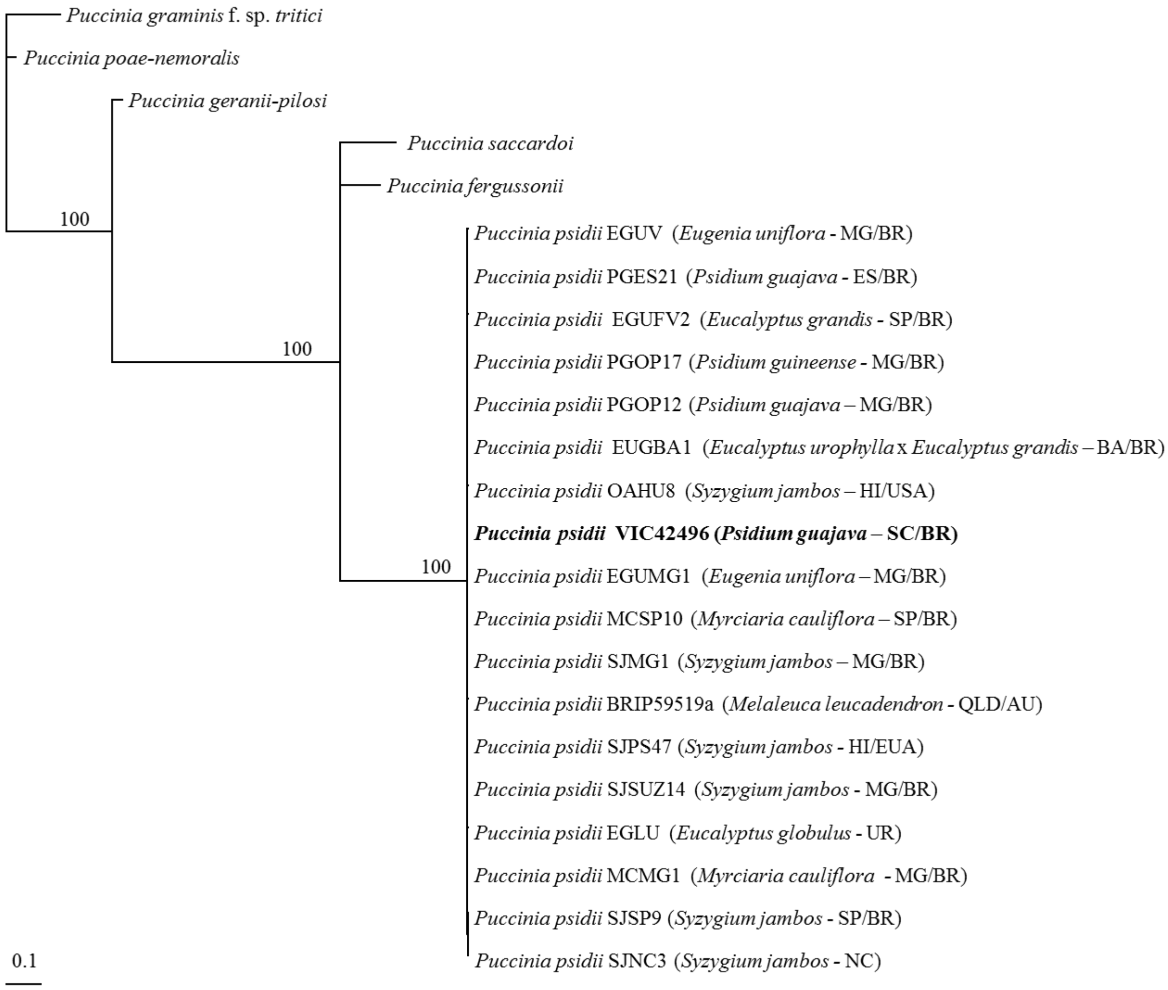

FIGURE 1 - Bayesian tree resulting from combined analysis of transcription elongation factor EF1-α, β-tubulin and ITS sequences for *Puccinia psidii* samples, including the epitype specimen **VIC42496** (bold font). The numbers above the branches indicate posterior probability percentage support. The tree was rooted to *Puccinia graminis* f. sp. *tritici*. In parenthesis hosts, states and countries of the *P. psidii* collections: Espírito Santo (ES), SP (São Paulo), Bahia (BA), Santa Catarina (SC), Queensland (QLD), Hawaii (HI), Brazil (BR), United States (USA), Australia (AU), Uruguay (UR) and New Caledonia (NC).

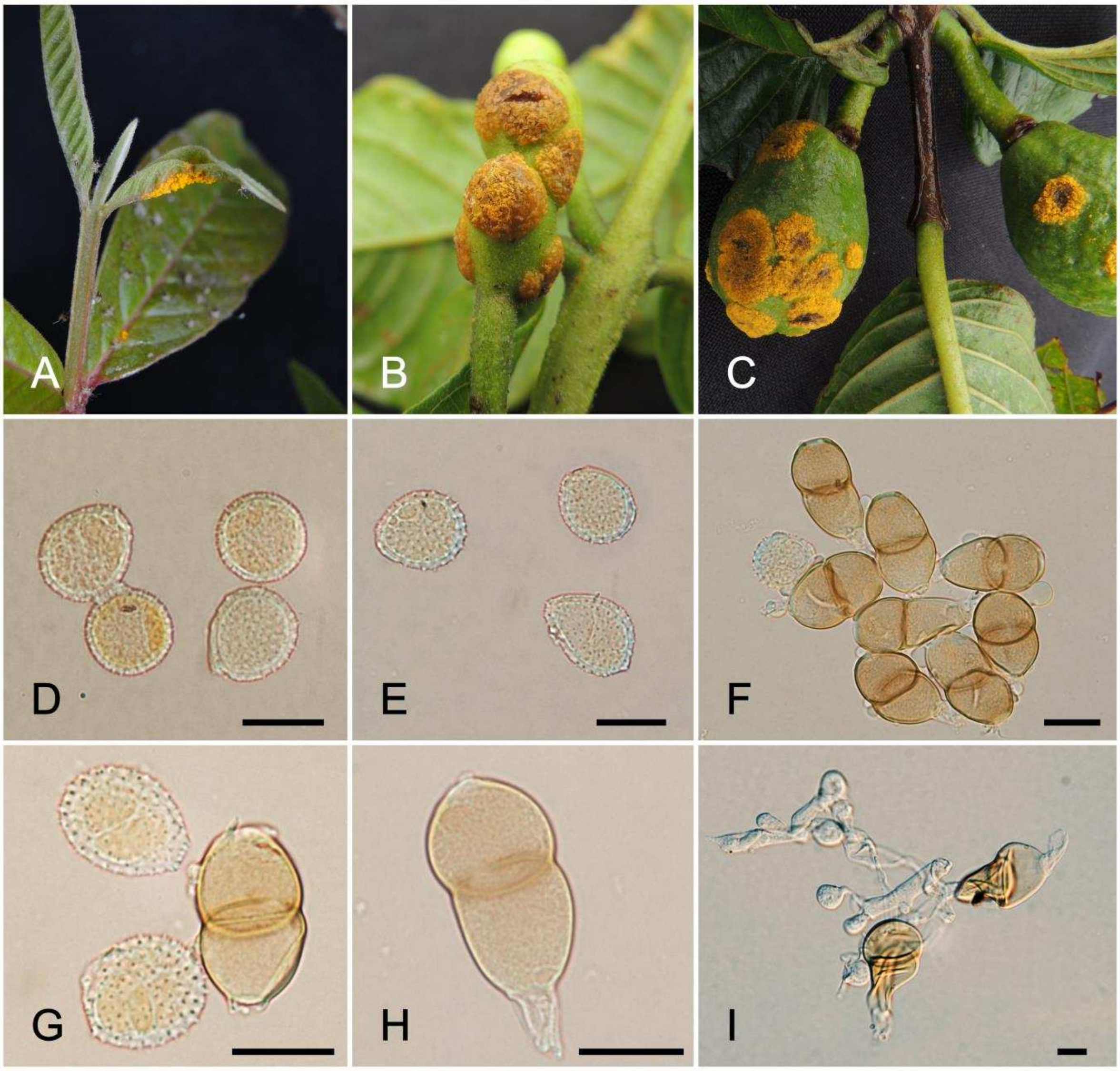

FIGURE 2 - *Puccinia psidii* (epitype – **VIC42496**). **A-C.** Leaves, buds and fruits infected with *P. psidii,* collected from *Psidium guajava* in Araquari. **D-E**. Urediniospore. **F-H.** Teliospores and urediniospores. **I.** Germinated teliospores and basidiospores. Bars = 20 µm.

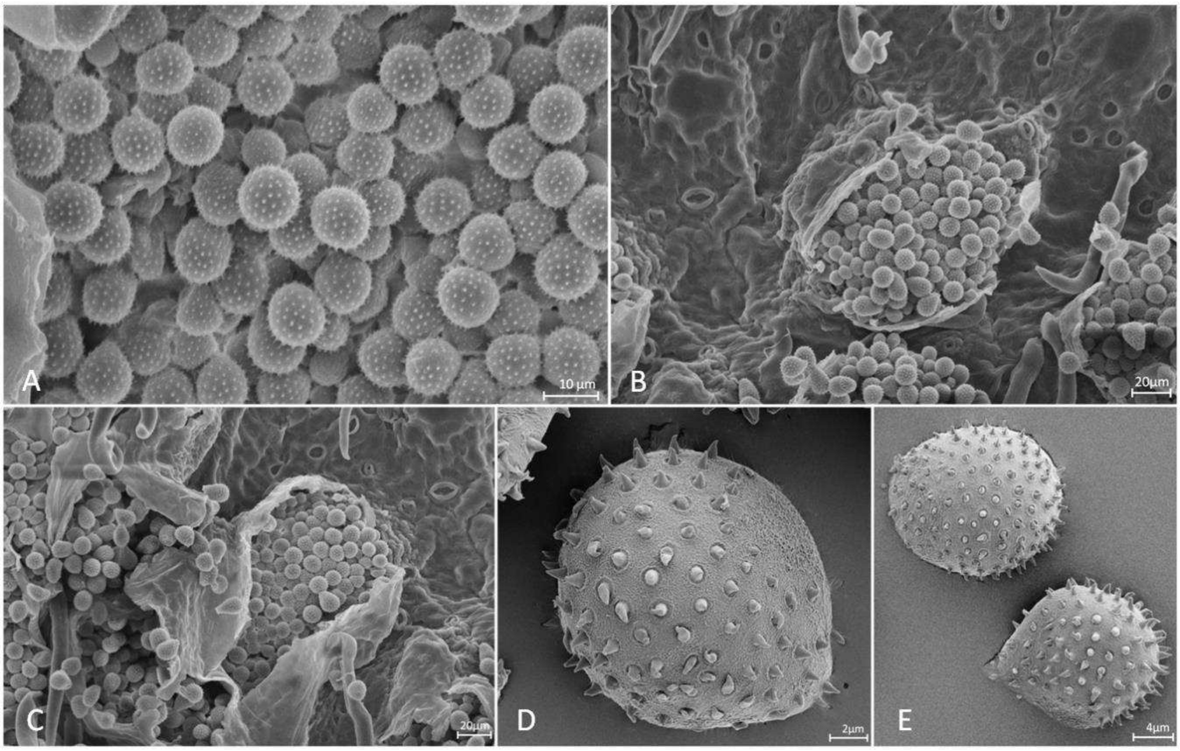

FIGURE 3 - Uredinia and urediniospores of *Puccinia psidii* (epitype – **VIC42496**). **A-C.** Uredinia and urediniospores on a *Psidium guajava* leaf. **D.** Tonsure (upper right) on urediniospore. **E.** Echinulate urediniospores.

ARTICLE 2

Formation, nuclear condition and infection of basidiospores of *Puccinia psidii* (Short Communication)

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Abstract

Puccinia psidii has been reported in 244 host species and 56 genera in the Myrtaceae. Due to its broad host range, massive urediniospore production and high capacity to be spread over long distances, the pathogen is a global threat to biodiversity of native species and exotic commercial plantations. Although it is considered an autoecious and macrocyclic rust, the pycnium stage is unknown and there is no information about the nuclear condition and infection of basidiospores. In this study, we evaluated the formation and nuclear condition of *P. psidii* basidiospores, collected on *Syzygium jambos* and *Eucalyptus grandis* and the infection process on *S. jambos* leaves. Germinated teliospores were observed using light and fluorescence microscope and electron microscope. After teliospore germination and nuclear fusion, the diploid nucleus migrated into the metabasidum and underwent a meiotic division resulting in four haploid nuclei. A septum was formed between each nucleus and resulted in four uninucleate cells. Shortly after, haploid nuclei underwent a mitotic division forming binucleate basidiospores. Of 200 basidiospore examined 68% were binucleate and 32% uninucleate. Germination of basidiospore and formation of rudimentary lobate apressoria were observed, but no penetration and infection were found on detached leaves of *S. jambos*.

Keywords: *Eucalyptus* spp., *Syzygium jambos*, Myrtaceae, rust, binuclear, Myrtle rust, Guava rust

Puccinia psidii Winter has been reported in 244 host species and 56 genera in the Myrtaceae. In the last years the pathogen has been of great concern in commercial eucalyptus plantations and also to native species of Myrtaceae in areas where the pathogen has recently been introduced, such as Hawaii, Japan, Australia, China and New Caledonia (Uchida et al. 2006, Kawanishi et al. 2009, Carnegie et al. 2010, Zhuang &Wei 2011, Giblin 2013).

Despite the intercontinental importance of this rust fungus and numerous studies especially on the *P. psidii* x *Eucalyptus* pathosystem (Xavier 1997, Xavier et al. 2001, Junghans et al. 2003a, Junghans et al. 2003b, Graça et al. 2011, Lana et al. 2012, Leite et al. 2013, Miranda et al. 2013), the lifecycle of the pathogen is still unclear. *Puccinia psidii* is apparently a macrocyclic and autoecious rust lacking the pycnium stage (Coutinho et al. 1998), although Simpson et al. (2006) also suggested that *P. psidii* could be heteroecious with an unknown alternate aecial host. However, using a special device ("Germinatélio") to generate only basidiospores, Figueiredo (2001) was able to infect seedlings of *Syzygium jambos* (L.) Alston. Aeciospores morphologically identical to urediniospores were produced 18 days after inoculation. But, it is possible that urediniospores contamination may have been the source of these infections. Thus, there is no conclusive evidence on the infectivity of basidiospores and their biological importance in the *P. psidii* lifecycle.

Germination of teliospores and subsequent formation of metabasidia and basidiospores are key structures to study the nuclear condition of *P. psidii*. In addition, information about the number of nuclei, karyogamy, meiosis and other nuclear cycle of events that take place in the telium phase are important because they are source of genetic variability, essential for the emergence of new races of the pathogen. Therefore, this study aimed to determine the nuclear behavior and infectivity of basidiospores of *P. psidii*.

Teliospores collected from infected *S. jambos* seedlings and *Eucalyptus grandis* Hill ex Maiden cuttings were transferred separately to excavated slides containing 100 µL of sterile distilled water in each cavity and incubated at 22°C in the dark for 0, 1, 2, 4, 6, 8 and 12h. After incubation, in each cavity the suspension was fixed in 100 µL of ethanol: acetic acid (v:v 1:3) solution. The samples were kept at 5°C until used for microscopic examination. For the observation of metabasidium, basidiospore and nuclear development, 10µL of fixed teliospore suspension were mixed with 5 µL of 0.001% SYBR Green I, prepared in 10 mmol/L KH_2PO_4 containing 18% glycerol and 1µg/mL of calcofluor (Campos & Costa, 2010). The preparation was observed with a Nikon E600 light microscope, using an excitation wavelength range of 450–520 nm and filter WU, and photographed with a Fujix HC-300Z digital camera. Two replicates were performed for each time.

The processes of adhesion, germination and penetration of basidiospores of *P. psidii* in *S. jambos* leaves were observed by scanning electron microscopy (SEM). Teliospore suspension was prepared and incubated for 12h until basidiospores production, as previously described. Petioles of not completely-expanded detached leaves were wrapped in water moistened cotton cloth to maintain their turgor. Drops (approximately 10µl) of basidiospore + teliospore suspension were placed on the abaxial leaves surface and incubated in a moisture chamber (Gerbox + moistened foam + nylon screen) at 22°C, in the dark for 12, 15, 24, 42 and 68h. For each time, six fragments of leaves (5 mm^2) were collected and fixed in 2.5% glutaraldehyde prepared in 0,1M phosphate buffer (pH 7.2) for 12h at 5°C. After fixing, fragments were washed three times at 10 min intervals in the same phosphate buffer and dehydrated in an ethanol series (30, 50, 70, 80, 95 and 100%), for 10 min for each concentration and dried using a critical point dryer (Bal-zers, model CPD030, BAL-TEC AG). The fragments were mounted on a metal support and metalized with a 150-Å thick gold using an Electron Microscopy Sciences 550x sputtercoater and examined using a scanning electron microscope (LEO, modelo 1430VP).

All the nuclear process observed were the same whether teliospores were collected on *E. grandis* or *S. jambos* leaves. Immature teliospores had two smaller nuclei per cell, less pigment with thinner wall as compared with mature teliospores (Figure 1A). Most of teliospores had only one nucleus suggesting that karyogamy occurs even before the onset of germination (Figure 1B). The period from teliospore germination until basidiospore formation and of all nuclear events varied according to the physiological state of telia. This lack of synchrony was also observed in cells of the same teliospore. Presence of abnormal structures due to excess humidity during incubation was not observed. Teliospores did not exhibit a dormant period and 1 to 2 h after germination a diploid nucleus migrated into the metabasidium (Figure 1C). The first meiotic division (meiosis I) occurred 4h after incubation (Figure 1D). After meiosis I a septum was formed between the two nuclei and then meiosis II was observed (Figure 1E). This last event was followed by the formation of two other septa, resulting in four haploid nuclei in each cell (Figure 1F). In some cases a mitotic division nuclear took place within the metabasidium and migration of one or two nuclei into basidiospore was observed (Figure 1G). Mitotic division was also observed within the basidiospores after migration of a single haploid nucleus. Sterigma and basidiospore formation started between 6 and 12h after incubation and there is no specific sequence for the formation of such structures.

After 12h of incubation, basidiospore formation and nuclear migration into basidiospores were complete (Figure 1H). The percentage of binucleate and uninucleate basidiospores was 68% and 32%, respectively. Mono and dikaryotic basidiospores detached from teliospore had already started to germinate (Figure 2 A-B). Basidiospore germ tubes arise from the appendage where the spore was attached to the sterigma (Figure 2C). Secondary basidiospores were not observed. Both mono and dikaryotic basidiospores germinated, however we did not observe the presence of two nuclei inside germ tubes of dikaryotic basidiospores.

Approximately 1500 basidiospores were evaluated by SEM. Degradation of host cuticle around the basidiospore germ tube was observed due to the release of a possible degrading enzyme during germination (Figure 2D). At 12h after inoculation rudimentary lobate appressorium were observed (Figure 2E). If *P. psidii* basidiospores are infective, the penetration probably is direct, since it was observed that the germ tube is capable of bypassing open stomata (Figure 2F). There were no differences in the size of the germ tube or appressorium formation at the different times of evaluation (Figure 2 C-G).

As observed for *P. psidii*, a binuclear condition of basidiospores is common among species of rusts belonging to Pucciniaceae (Anikster, 1983; Narisawa et al., 1993; Ono, 2002; Anikster et al., 2003). However, this feature does not explain the mechanisms of dikaryotization that probably occurs in other ways than by spermogonial stage (Allen, 1933; Narisawa et al. 1993; Cummins & Hiratsuka, 2003). The methodology used in this study for nuclei observation is simple and fast allowing cytological studies of other species of rust and other pathogens.

The images from SEM did not showed evidences of infectivity of basidiospores. Recently, other study (Morin et al., 2014) tried to elucidate the lifecycle of *P. psidii* but no sign of penetration of basidiospores was observed on leaves of *Agonis flexuosa* or *S. jambos*, as found in the present work.

Until now is not clear if basidiospores of *P. psidii* are non-infective or was not found an adequate methodology to prove this hypothesis. To prove the infectivity of basidiospores additional studies are necessary using electron transmission microscopy and cross sections of inoculated leaves to analyze intercellular basidiospore hyphae within the host, or maybe to find infection structures within the epidermal cells. To demonstrate the full life cycle of *P. psidii*, basidiospore infection and subsequent pustule development need to be observed.

Acknowledgements

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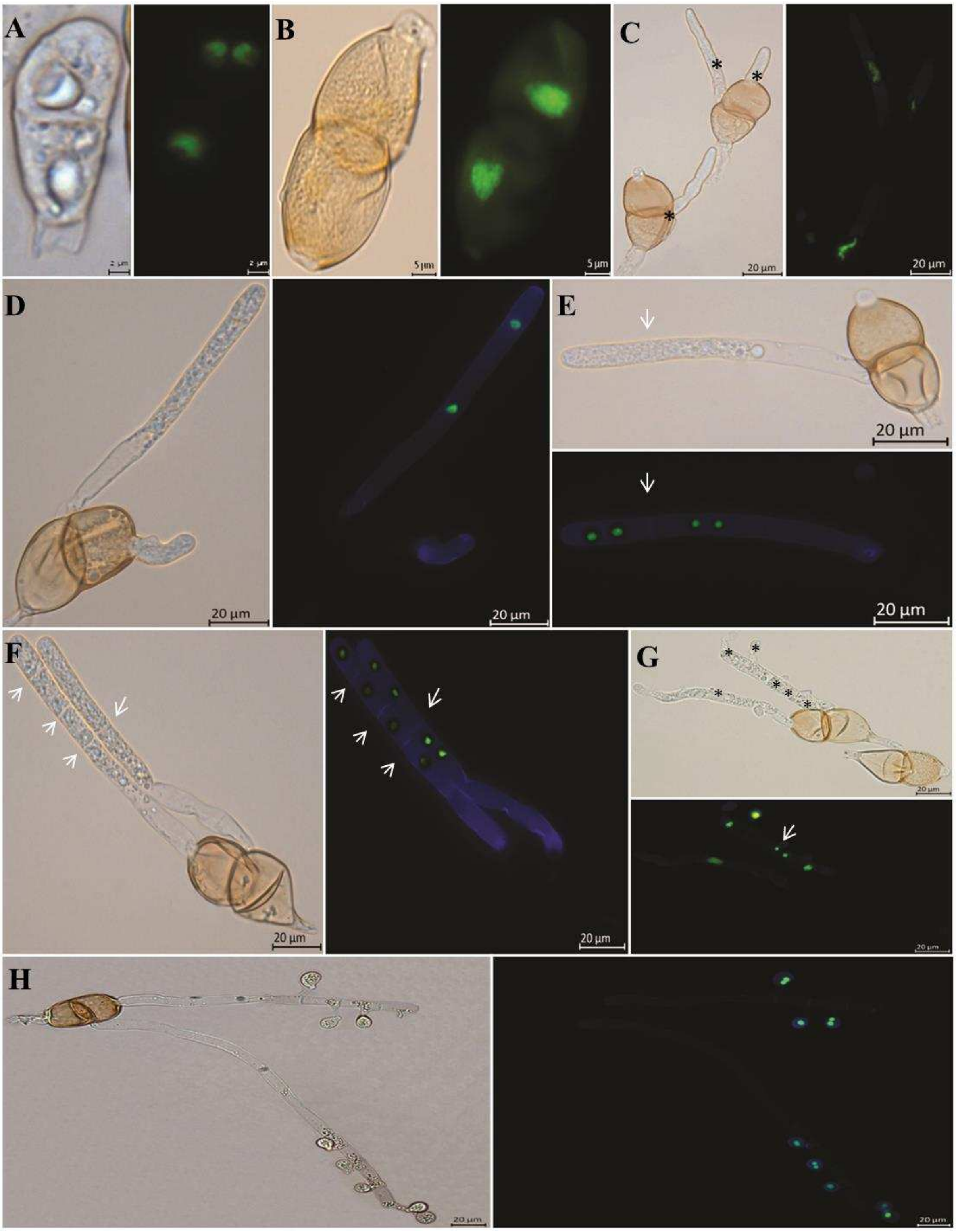

Figure 1 - Light and fluorescence microscopy showing the process of nuclear migration from germination of teliospores until the formation of basidiospores of *Puccinia psidii*; **A.** Immature teliospore containing two small nuclei in the upper cell; **B.** Mature teliospore containing a large nucleus in each cell; **C.** Begining of teliospore germination and migration of the nucleus into metabasidium (Asterisks indicate the position of the nucleus); **D.** Meiosis I

within metabasidium in the lower cell; **E.** - Meiosis II and formation of the first septum (arrow); **F.** Meiosis II in lower metabasidium and four cells separated by septum (arrow) containing one haploid nuclei each within upper metabasidium; **G.** Metabasidium showing nuclear development as basidiospore development initiates, with one basidiospore having a single nucleus and one cell of the metabasidium with two nuclei (arrow) (Asterisks indicate the position of the nucleus); **H.** Basidiospores with mono and binuclear.

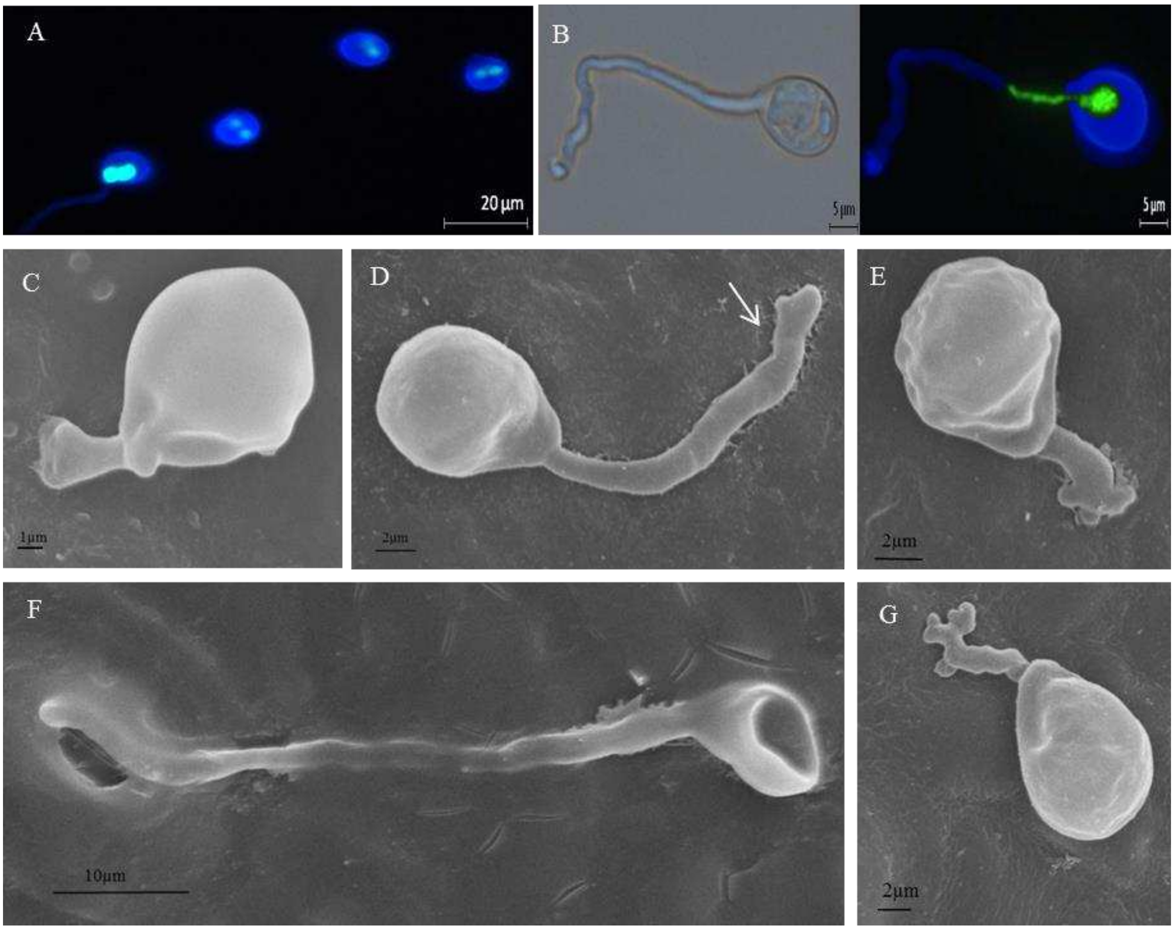

Figure 2 - Fluorescence microscopy and scanning electron microscopy pictures showing basidiospore germination. **A.B.** Germination of basidiospores and nucleus migration into germ tube; **C.** Germination of basidiospore from the appendage which the spore was attached to the sterigma; **D.** Degradation of the leaf cuticle (arrows) around germ tube.; **E.** Formation of rudimentary lobate appressorium 12h a.i.; **F.** Basidiospore germ tube bypassing a stomata; **G.** Basidiospore germinating 24h a.i., with rudimentary lobate appressorium.

ARTICLE 3

Microsatellite analysis of *Puccinia psidii* population in Australia

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Abstract

Puccina psidii is considered a biosecurity threat in Australia where species of Myrtaceae are dominant in many ecosystems. Since its first reported in the country, in April 2010, there has been little information about the population structure of the pathogen. In this study, six microsatellite loci were analysed to determine the genetic relationship among rust collections from different hosts and locations in Australia, New Caledonia and China. The Chinese and New Caledonian specimens share a multi-locus genotype with the majority of the Australian collections. At present, *P. psiidi* population in Australia is genetically uniform except for a few minor point mutations of the dominant genotype noted in only four of the 79 isolates examined.

Keywords: Myrtaceae, microsatellites, rust, plant disease, genotyping

Introduction

Australia is the centre of origin of eucalypt and melaleuca biodiversity (Ladiges *et al*. 2003, Crisp and Cook. 2013) and has over 1,646 species of Myrtaceae (Australian Flora Statitistic, https://www.anbg.gov.au/aust-veg/australian-flora-statistics.html).Plants belonging to the Myrtaceae are dominant in Australia in ecosystems ranging from tall forests to swamps and wetlands (Australian Government Department of Agriculture). *Puccina psidii*, known colloquially as guava rust, eucalyptus rust or myrtle rust, is considered a biosecurity threat to many of these ecosystems. The pathogen was first reported in Australia in April 2010, on *Agonis flexuosa* on the Central Coast of New South Wales (Carnegie *et al*. 2010) and spread rapidly along the east coast where it was detected in Queensland in December 2010 and one year later in Victoria (Pegg *et al.* 2014).

Up to now, the pathogen has been reported on about 56 genera and 244 species of host in the Myrtaceae distributed in different continents: from South and North America (Carnegie and Lidbetter 2012), Asia (Kawanishi *et al*. 2009, Zhuang and Wei 2011), Oceania (Carnegie *et al*. 2010, Giblin 2013) and Africa (Roux *et al*. 2013). After being introduced into new areas, *P. psidii* quickly expanded its host range as observed in Jamaica, Florida, Hawaii and Australia (MacLachlan 1938; Rayachhetry *et al*. 2001; Loope 2010; Pegg *et al*. 2013). In Brazil, the pathogen is considered endemic (Tommerup *et al*. 2003) and is not usually severe on native hosts with the exception of occasional epidemics in guava orchards (de Goes *et al*. 2004; Ribeiro and Pommer 2004), but it is a problem in eucalypt, an important industrial crop for the country (Alfenas *et al*. 2009).

The ultimate impact of the pathogen on Australian biodiversity is yet to be determined, but considering its rapid dissemination, wide host range and the severe damage reported to some species such as *Rhodamnia rubescens* (Benth.) Miq., *Rhodomyrtus psidioides* (G.Don) Benth., *Syzygium anisatum* (Vickery) Craven & Biffen and *Melaleuca quinquenervia* (Cav.) S.T.Blake (Carnegie & Cooper, 2011), *P. psidii* can put at risk not only the vegetation but also animal species which may depend on native plant species (Tommerup *et al*., 2003; Glen *et al*., 2007). Besides biodiversity, the pathogen can have a commercial impact on the forest, Lemon myrtle tea farms and timber industry (Plant Health Australia 2007) and commercial nurseries (Plant Health Australia 2010).

Since the pathogen was reported in 2010, studies have been conducted to identify vulnerable areas (Booth and Jovanovic, 2012; Elith *et al*. 2013) and the host range of susceptible and resistant plant species (Carnegie and Lidbetter 2012). However, there is no information about genetic variation of the pathogen population, how the rust was introduced into the country and how it spreads. Recent studies using microsatellite markers have determined the population structure and the host specificity of *P. psidii* in different areas such as Hawaii and Brazil (Zhong *et al*. 2011, Graça *et al*. 2013). In Hawaii, *P. psidii* isolates that are genetically uniform might have derived from the same strain originally introduced into the state. In Brazil, host species provides strong selection pressure on *P. psidii* populations, regardless of geographic location. Principal coordinate analysis also indicated a high degree of genetic differentiation among isolates collected on different host species, revealing five major groups, the first formed by collections from *Eucalyptus* spp. and *Syzygium jambos*, the second from *Psidium guajava* and *Psidium guineense* isolates, and three weakly separated groups formed by collections from *Syzygium cumini*, *Myrciaria cauliflora* and *Eugenia uniflora* (Graça *et al*. 2013). The existence of host-specific genotypes may indicate the occurrence of cryptic species within the *P. psidii* complex or maybe the pathogen is co-evolving to the level of "formae speciales" or even to species level. Genetic analysis of pathogen populations is required to understand the mechanisms generating genetic variation, host-pathogen coevolution, and in the management of resistance (Keiper *et al*. 2003). In this study microsatellite loci were analysed to determine the genetic relationship among rust isolates collected in different hosts and locations from Australia and recent incursions in other countries.

Material and methods

Sampling

A total of 79 single uredinial pustules of *P. psidii* were collected on 47 Myrtaceae species in Australia, New Caledonia (Figure 1) and China (Table 1). All survey points were geo-referenced and most collections were deposited in the Queensland plant pathology herbarium (BRIP). A portion of each collection was preserved in ethanol and retained for DNA extraction. Single pustules were excised and placed separately into 1.5-mL Eppendorf® tubes and stored at -80ºC prior to DNA extraction. Samples from New Caledonia consisted of urediniospores collected from multiple *Syzygium jambos* plants in New Caledonia were preserved in 70% ethanol and imported into Australian under import permit IP13103123. For the isolate from China, the DNA from a portion of HMAS242567 was extracted and imported under IP13007011.

DNA extraction

Genomic DNA was extracted directly from a single *P. psidii* pustule (fungus + host tissue) using one metal bead placed in a 1.5-ml microcentrifuge tube followed by two rounds of maceration using a TissueLyser II (Qiagen) for 2 min at the frequency 30 Hz. A total of 250 µl extraction buffer (Raeder and Broda 1985) was added and the tubes incubated at 65°C for 1 h. Tubes were centrifuged at 14,000 rpm for 15 min and the supernatant removed. DNA was purified by binding to silica (Boyle and Lew 1995). Briefly, 600 µl of 100% NaI and 10 µl silica were added to 200 µl of the supernatant and vortexed briefly. The mixture was incubated on ice for 15 min with occasional shaking. Tubes were centrifuged for 10 s at 14,000 rpm, the supernatant removed, and the pellet resuspended in 600 µl of wash buffer (100 mM NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA in 50% ethanol). Following centrifugation for 10 s at 14,000 rpm, the supernatant was removed, the pellet suspended in 600 µl 100% ethanol and centrifuged as before. Finally, the supernatant was removed and the pellet dried for 20 min. DNA was eluted by adding 20 µl of TE buffer, vortexing briefly and incubating at 45°C for 10 min. Supernatant containing DNA was removed following centrifugation for 2 min at 14,000 rpm and stored at –20°C.

Microsatellite genotyping

The samples were genotyped at 6 microsatellite loci (EF523503, EF523504, EF523507, EF523508, EF523510, EF523513) (Zhong *et al*. 2008, Graça *et al*. 2013). For each 10 µL PCR reaction we used 5 µL of 2x Master Mix (Type-It Microsatellite PCR kit, Qiagen), 0.1µL (20 µM) of forward (labelled with either D2, D3 or D4 Well-RED fluorescent dye, Sigma-Aldrich) and reverse primers, 0.2 mg/mL of Bovine Serum Albumin (BSA, Fisher BioReagents™), 3.6 µL of nuclease-free water and 1 µL genomic DNA. PCR amplifications were performed using a thermal cycler (model 2720, Applied Biosystems) and the following program: 95ºC for 3 min, then 34 cycles of 94°C for 15s, 45 to 50°C (depending on the locus) for 15s, 72°C for 45s, ending with a hold at 60 °C for 30 minutes, then 14°C. Fragment analysis was conducted on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter), using 1 µL of PCR product mixed with 38.5 µL Sample Loading Solution (Beckman Coulter) and 0.5 µL size marker (DNA Size Standard Kit – 400, Beckman Coulter).

Results

Small genetic variability was found among the 79 isolates of *P. spidii*, a single genotype was observed in the majority of collections (Table 2). However, in four isolates from Australia (MR40, MR44, MR58 and MR60) an unusual allele was detected in tree loci (Table 2). The four isolates were collected in Cairns and neighborhood on three different hosts (Table 2). Genotyping was repeated 2-3 times for these collections from the original DNA, providing the same result each time. Further DNA was extracted from additional pustules from the same collections, with variable genotyping results (Table 2). In two instances, further variation at the variable locus was detected; in the third, the common MLG was detected.

Discussion

Low genetic variation was demonstrated in *P. psidii* isolates from Australia, New Caledonia and China using six polymorphic and representative microsatellites to access the genetic variability among different *P. psidii* populations (Zhong *et al.* 2008, Zhong *et al*. 2011, Graça *et al.* 2013). The same heterozygous genotype found among the majority of isolates indicates no genetic recombination and no selection by host species. These results show that there was a recent introduction of a single rust genotype in Australia. Although teliospores were identified in 20% of the samples in a survey in Queensland (Pegg *et al.* 2013), the lack of recombination and structure of Australian isolates is consistent with the lack of recombination in the Hawaiian rust population (Zhong *et al*. 2011, Graça 2011), where the pathogen was reported nine years ago (Uchida *et al*. 2006). The low variability in the Australian population is consistent with clonal reproduction, precluding analysis with GenAlex 6.4 (Peakall & Smouse 2006). Isolates that showed an unusual allele size were collected in Cairns and neighborhood on three different hosts and it was not found any correlation among isolates, host species, allele size and loci that may indicate random mutation. Similar levels of mutation have been observed in clonal populations of *Puccinia triticina* in wheat cultivars (Goyeau *et al.* 2007). Besides that, rust fungi cannot be readily grown in axenic culture what makes possible an eventual polymorphism arising from contaminant DNA from two different strains in the same pustule (Keiper et al. 2003).

Microsatellite markers have been used to infer the origin of the *P. psidii* incursion in Hawaii. A unique genotype found in four Hawaiian Islands (Maui, Oahu, Kauai, and Big Island) was also found in two isolates from different hosts in California, indicating that California may have been the source of the *P. psidii* introduction into the Hawaii, probably by the trade of Myrtaceae plant between both states as hypothesized by Graça (2011). However, the origin of the genotype in California is unknown.

The collections from New Caledonia and China and the majority of Australian collections have the same genotype as that present in Hawaii. Although *P. psidii* was reported first in Hawaii followed by China, Australia and most recently, New Caledonia, it is not possible to confirm the origin of the incursion in these countries, unlike in Japan where the rust was detected on *Metrosideros* plants imported from Hawaii (Kawanishi et al, 2009). The rust may have been distributed from California to all of the other countries or may have travelled from e.g., California to Hawaii, from Hawaii to Australia, from Australia to New Caledonia.

In contrast with the rust population in Australia and Hawaii, the genetic variability of *P. psidii* collections in Brazil is high. In a recent study 10 microsatellite loci were analyzed for 148 *P. psidii* isolate collected from seven host species (Graça et al. 2013). All loci were polymorphic and host species provide strong selection on *P. psidii* populations regardless of geographic location. Although the *P. psidii* population has high genetic variability and the pathogen is widely distributed in

Brazil, the genotype present in Hawaii and California has not been detected in Brazil (Graça 2011).

The source and pathway of the incursion in Australia is unknown. Although the country has a continental size, wind combined with susceptible host and suitable climatic conditions provided a near-contiguous corridor where the spores of the pathogen were spread along the east coast (Carnegie and Lidbetter, 2012). There are also evidence of aerial dispersal of two other rust species, *Melampsora larici-populina* Klebah and *Melampsora medus*a Thümenth, from the east coast of Australia to New Zealand across the Tasman Sea (Close et al. 1978). Is not known if the same happened with *P. psidii* between Australia and New Caledonia. Besides airflows, commercial trade of plants and movements of people and commodities are likely to be the other long-distance dispersal pathways for pathogen spores (Sheridan 1989; Williams et al. 2000).

Artificial inoculations showed that at least 107 host native species in 30 genera are susceptible to this predominant genotype (Carnegie and Lidbetter 2012). The isolates from Australia were collected from July to Augustus 2013. Three years after the first report of the pathogen into the country, a few mutants of the dominant genotype were already observed. The genetic structure of the isolates observed at present can be very different in a few years or even decades after the introduction of any new *P. psidii* genotypes or a beneficial mutation that increases fitness of the pathogen in a particular host and environment, which represents a potential risk. Thus, avoiding the introduction of new *P. psidii* genotypes into, and dispersal around the country in areas of high Myrtaceae biodiversity which have not previously been exposed to this rust, is highly desirable.

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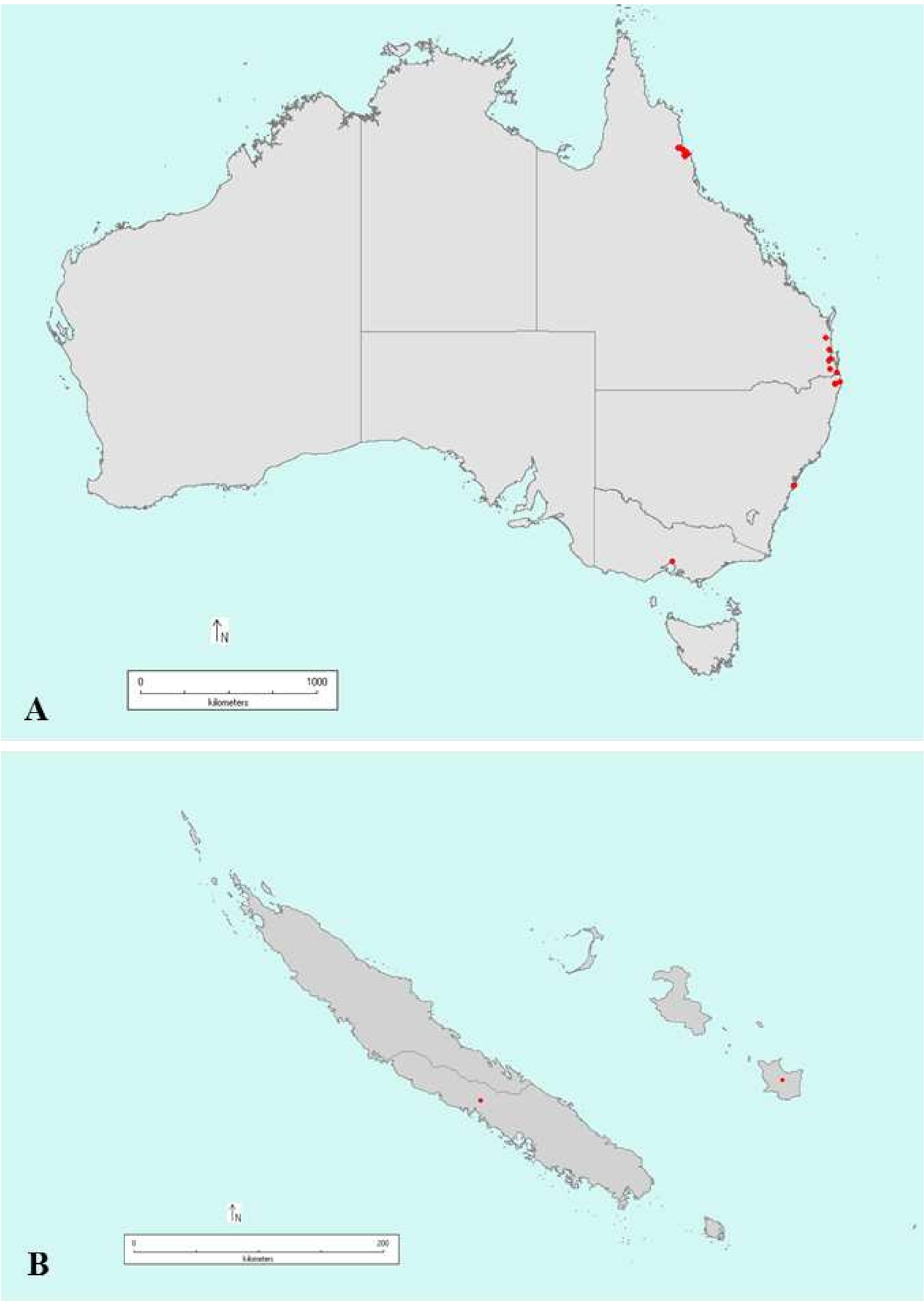

Figure 1 – Sampling locations of *Puccinia psidii* in Australia (A) and in New Caledonia (B)

Table1 - Host and geographic origin of *Puccinia psidii* isolates

Sample	**Host**	**Location**	**Latitude**	**Longitude**
MR1	*Syzygium jambos*	NSW/Australia	-33,868	151,207
MR2	*Syzygium jambos*	QLD/Australia	-27,919	153,051
MR3	*Syzygium australe*	QLD/Australia	-27,475	152,973
MR4	*Rhodomyrtus canescens*	QLD/Australia	-27,475	152,973
MR5	*Rhodomyrtus pervagata*	QLD/Australia	-27,475	152,973
MR6	*Syzygium nervosum*	QLD/Australia	-27,475	152,973
MR7	*Syzygium macilwraithianum*	QLD/Australia	-27,475	152,973
MR8	*Rhodamnia blairiana*	QLD/Australia	-27,475	152,973
MR9	*Chamelacium uncinatum*	QLD/Australia	-27,475	152,973
MR10	*Backhousia oligantha*	QLD/Australia	-27,475	152,973
MR11	*Austromyrtus dulcis*	NSW/Australia	-28,605	153,572
MR13	*Melaleuca quinquenervia*	NSW/Australia	-28,605	153,572
MR14	*Rhodomyrtus psidioides*	NSW/Australia	-28,605	153,572
MR16	*Agonis flexuosa*	NSW/Australia	-28,672	153,279
MR18	*Syzygium jambos*	QLD/Australia	-27,485	152,992
MR19	*Melaleuca fluviatilis*	QLD/Australia	-27,404	153,073
MR20	*Melaleuca quinquenervia*	QLD/Australia	-26,333	152,82
MR21	*Rhodamnia rubescens*	QLD/Australia	-26,333	152,82
MR22	*Melaleuca quinquenervia*	QLD/Australia	-26,941	152,974
MR23	*Leptosperma trinervium*	QLD/Australia	-26,941	152,974
MR24	*Melaleuca quinquenervia*	QLD/Australia	-26,941	152,974
MR25	*Melaleuca quinquenervia*	QLD/Australia	-26,941	152,974
MR26	*Rhodamnia sessiliflora*	QLD/Australia	-27,494	152,944
MR27	*Eugenia reinwardtiana*	QLD/Australia	-16,912	145,767
MR28	*Melaleuca quinquenervia*	QLD/Australia	-17,222	145, 664
MR29	*Eugenia reinwardtiana*	QLD/Australia	-17,222	145, 664
MR30	*Syzygium or Eugenia ??*	QLD/Australia	-17,222	145, 664
MR31	*Melaleuca* sp.	QLD/Australia	-17,222	145, 664
MR32	*Gossia* sp.	QLD/Australia	-16,816	145,686
MR33	*Eugenia reinwardtiana*	QLD/Australia	-16,816	145,686
MR34	*Melaleuca leucadendron*	QLD/Australia	-16,816	145,686
MR35	*Austromyrtus dulcis*	QLD/Australia	-16,816	145,686
MR36	*Gossia inophloia*	QLD/Australia	-16,816	145,686
MR37	hybrid *Syzygium leuhmannii* x *S. wilsonii*	QLD/Australia	-16,816	145,686
MR38	*Leptospermum* sp.	QLD/Australia	-16,816	145,686
MR39	*Xanthostemon* sp.	QLD/Australia	-16,816	145,686
MR40	*Gossia myrsinocarpa*	QLD/Australia	-16,812	145,685
MR41	*Syzygium cormiflorum*	QLD/Australia	-16,812	145,685
MR42	*Rhodamnia sessiliflora*	QLD/Australia	-16,812	145,685
MR43	*Gossia* sp.	QLD/Australia	-16,812	145,685
MR44	*Rhodamnia spongiosa*	QLD/Australia	-16,812	145,685
MR45	*Leptospermum madidum subsp. sativum*	QLD/Australia	-16,820	145,642

MR46	*Tristaniopsis exiliflora*	QLD/Australia	-16,820	145,642
MR47	*Leptospermum petersonii*	QLD/Australia	-16,825	145,624
MR48	*Syzygium wilsonii* subs wilsonii	QLD/Australia	-16,825	145,624
MR49	*Melaleuca viminalis*	QLD/Australia	-16,825	145,624
MR50	*Gossia* sp.	QLD/Australia	-16,825	145,624
MR51	*Eugenia reinwardtiana*	QLD/Australia	-16,825	145,624
MR52	*Rhodamnia sessiliflora*	QLD/Australia	-17,037	145,613
MR53	*Rhodamnia* sp.	QLD/Australia	-17,037	145,613
MR54	*Gossia myrsinocarpa*	QLD/Australia	-17,037	145,613
MR55	*Syzygium sayeri*	QLD/Australia	-17,037	145,613
MR56	*Melaleuca viridiflora*	QLD/Australia	-16,980	145,552
MR57	*Melaleuca* sp.	QLD/Australia	-16,980	145,552
MR58	*Syzygium jambos*	QLD/Australia	-16,876	145,757
MR59	*Syzygium kuranda*	QLD/Australia	-16,831	145,667
MR60	*Gossia myrsinocarpa*	QLD/Australia	-16,804	145,636
MR61	*Tristaniopsis exiliflora*	QLD/Australia	-16,804	145,636
MR62	*Rhodamnia spongiosa*	QLD/Australia	-16,804	145,636
MR63	*Decaspermum humile*	QLD/Australia	-16,804	145,636
MR64	*Rhodomyrtus pervagata*	QLD/Australia	-16,796	145,622
MR65	*Eucalyptus pellita*	QLD/Australia	-16,699	145,529
MR66	*Melaleuca nervosa*	QLD/Australia	-16,699	145,529
MR67	*Backhousia hughesii*	QLD/Australia	-16,681	145,519
MR68	*Melaleuca nervosa*	QLD/Australia	-16,658	145,477
MR69	*Rhodomyrtus effusa*	QLD/Australia	-16,582	145,321
MR70	*Rhodomyrtus pervagata*	QLD/Australia	-16,582	145,321
MR71	*Gossia lewisensis*	QLD/Australia	-16,594	145,284
MR72	*Rhodomyrtus canescens*	QLD/Australia	-16,588	145,275
MR73	*Syzygium apodophyllum*	QLD/Australia	-16,898	145,747
MR74	*Myrtus communis*	VIC/Australia	-37,807	144,953
MR75	*Melaleuca quinquenervia*	QLD/Australia	-25,191	153,151
MR77	*Rhodamnia maideniana*	QLD/Australia	-28,209	153,270
MR78	*Syzygium jambos*	QLD/Australia	-28,209	153,270
NC3	*Syzygium jambos*	Farino/NC	-21,667	165,767
NC4	*Syzygium jambos*	Farino/NC	-21,667	165,767
NCC	*Syzygium jambos*	Farino/ NC	-21,667	165,767
NCE	*Eugenia gacognei*	Mare/ NC	-22,016	166,033
Ppchina	*Syzygium jambos*	Hainan/China	20,167	110,250

[a] NSW= New South Wales, QLD= Queensland, NC= New Caledonia

Table 2 – Allele sizes for microsatellite loci of isolates collected in Australia (MR), New Caledonia (NC) and China (PpChina). In bold unexpected allele size detected in different pustules from the same isolate.

Collection	Locus					
	503	504	507	508	510	513
MR1[a]	217, 219	155, 157	162, 171	172, 178	68, 78	215, 227
NC3[b]	217, 219	155, 157	162, 171	172, 178	68, 78	215, 227
PpChina 1	217, 219	155, 157	162, 171	172, 178	68, 78	215, 227
MR40 (pustule 1)	**215, 217**	155, 157	162, 171	172, 178	68, 78	215, 227
MR40 (pustule 2)	**213, 215**	155, 157	162, 171	172, 178	68, 78	215, 227
MR40 (pustule 3)	217, 219	155, 157	162, 171	172, 178	68, 78	215, 227
MR44 (pustule 1)	217, 219	155, 157	162, 171	172, 178	68, 78	**215, 221**
MR44 (pustule 2)	217, 219	155, 157	162, 171	172, 178	68, 78	**215, 223**
MR58 (pustule1)	217, 219	155, 157	162, 171	**175, 177**	68, 78	215, 227
MR58 (pustule2)	217, 219	155, 157	162, 171	**177, 179**	68, 78	215, 227
MR60 (pustule 1)	217, 219	155, 157	162, 171	172, 178	68, 78	**215, 215**
MR60 (pustule 2)	217, 219	155, 157	162, 171	172, 178	68, 78	215, 227

[a] MR1 and majority isolates (=70) from Australia have the same genotype

[b] All isolates from New Caledonia have the same genotype

ARTICLE 4

Genetic and physiological variability of *Puccinia psidii* isolates

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Abstract

Cross-inoculation and microsatellite markers have indicated ecological specialization of *Puccini psidii* isolates. The aim of this study was to investigate the correlation between genetic and physiological variability within *P. psidii* population. Eighty eight isolates of *Puccinia psidii* collected on 20 host species in five Brazilian states were genotyped using six microsatellites. Among all genotyped isolates, 50 were used for cross-inoculation on a set of six Myrtaceae plants. In general, genotypes were associated according to the host species, regardless the location. The cross-inoculation results varied according to isolate-host tested. Although the correlation was very small, the Mantel tests ($p< 0.01$, $r= 0.061$) showed an association between the Euclidean distances of the physiological groups with the genetic distance matrices. Complete match between them was not found.

Keywords: Cross-inoculation, Myrtaceae, rust, microsatellite

Introduction

Puccinia psidii, guava rust, was originally described in 1884 in *Psidium guajava* (=*Psidium pomiferum* L.) in São Francisco do Sul, Santa Catarina state, Brazil (Winter, 1884). Many years latter, in 1944, *P. psidii* was reported in *Corymbia citriodora* (Hook) K.D. Hill & L.A.S. Johnson by Joffily in Itaguai, Rio de Janeiro. The disease remained endemic until 1973, when a rust outbreak occurred in nurseries and plantations of *Eucalyptus grandis* Maiden (South African province) in Espírito Santo state (Ferreira, 1983).

Puccinia psidii has a broad host range infecting approximately 56 genera and 244 species in the Myrtaceae. The pathogen has been reported in different countries around the world (Kawanishi *et al*. 2009, Zhuang and Wei 2011, Carnegie and Lidbetter 2012, Giblin 2013, Roux *et al*. 2013) and is considered a global threat to exotic commercial crops and biodiversity of native species. In Jamaica, rust disease caused by this pathogen devastated plantations of *Pimenta dioica* (MacLachlan, 1938); in Florida the pathogen severely infected plants of *Pimenta dioica* and *Melaleuca quinquernevia* (Rayachhetry et al., 1997). In Brazil, the rust is a severe disease in some susceptible clonal plantations of eucalyptus, infecting plants in nursery and field conditions (Alfenas et al., 2009). Although only few studies have reported the effects of the disease on growth and productivity, wood volume losses of up to 40% in eucalypt plantations in São Paulo have been attributed to *P. psidii* infection (Takahashi, 2002). In Hawaii, rust infects *Meterosideros* spp., a plant species that comprises over 80% of the remaining native forests of the Hawaiian Islands (Uchida et al., 2006). In Australia, where the pathogen was recently introduced and Myrtaceae species are predominant in many native ecosystems, *P. psidii* has been found on over 100 species and 30 genera (Carnegie & Lidbetter, 2012).

Integrated management of the disease, including breeding for resistance, can only be successful if it is based on knowledge of the variability of the pathogen population. For several years, cross inoculations on different species of Myrtaceae have been used to identify pathotype groups in *P. psidii* populations. Although the results are conflicting, all indicated physiological specialization within P. *psidii* (MacLachlan 1938, Marlatt & Kimbrough 1979, Castro et al., 1983, Coutinho & Figueiredo, 1984, Ferreira, 1981, Rayachhetry et al. 2001, Coelho et al., 2001, Xavier 2002, Aparecido et al., 2003b). According to Ferreira (1981), spores collected from wild and commercial guava did not infect *Syzygium jambos* (L.) Alston, *Eucalyptus* spp., *Myrciaria cauliflora* (Mart.) O. Bergand and *Callistemon* spp. However, Castro et al. (1983) showed that guava isolates infect *E. grandis* clones. Coelho et al. (2001) presented three physiological groups of *P. psidii*. Group 1, spores collected on *Eucalyptus* spp., *S. jambos* and *Myrcia itambensis* O. Berg infected *E. grandis* and *S. jambos*; group 2 spores from guava infected eucalyptus and guava, and group 3 spores from guava infected only the original host. Aparecido et al. (2003b) identified four physiological groups of *P. psidii* by inoculations onto five host species. Group 1 spores collected from *S. jambos* and *E. grandis* infected *S. jambos* and *Corymbia citriodora*, group 2 spores from guava infected the original host and *C. citriodora*, group 3 spores collected from *M. cauliflora* infected *S. jambos*, *C. citriodora* and *Eugenia involucrata* DC., and group 4 spores from *Eugenia cambucae* Mattos infected only *E. involucrata.*

Recently, microsatellite markers have indicated a likely selection of *P. psidii* by host species, regardless of geographic location (Graça et al., 2013). The subdivision of the pathogen population was also confirmed by principal component analysis indicating a high degree of genetic differentiation among isolates collected from different hosts. Five main groups were detected; the first included isolates from *Eucalyptus* and *S. jambos*, the second comprised isolates from *P. guajava* and *P. guineense* and other three groups were collected from *Syzygium cumini* (L.) Skeels, *M. cauliflora* and *E. uniflora,* respectively.

Although cross inoculation and molecular studies of *P. psidii* indicated a possible physiological specialization, both studies were realized separately using different isolates and hosts. Thus, the aim of this study was to investigate the relationship between genetic and physiological variability of different isolates of *P. psidii*.

Material and methods

Sampling

Eighty eight isolates of *P. psidii* were collected from leaves and fruits in 20 species of Myrtaceae in five Brazilian states (Figure 1). Each isolate was geo-referenced and spores collected on the same plant were considered as a single isolate. Two types of sampling were made: i) Single uredinial pustules ca. 5mm diameter (containing spores and tissue host) were collected separately in 1.5-mL Eppendorf® tubes and stored at -80ºC prior to DNA extraction and ii) Infected leaves were collected and spores from multiple leaves of the same plant were harvested using a soft brush and stored at -80ºC prior to inoculation.

Cross-inoculation

The spores were inoculated on a set of six Myrtaceae plants: *Eucalypus urophylla* (clone CLR 079), *Syzygium jambo* L., *Psidium guajava* L., *Myrciaria cauliflora* Berg, *Eugenia uniflora* Berg, and *Psidium guineense* SW. Seedlings of each species were maintained in 2-L plastic bags containing the substrate MecPlant®

(partially decomposed pine bark and vermiculite), supplemented with 3 Kg/m3 of Osmocote® and 8 Kg/m3 of simple super phosphate. From each plant, 4-5 healthy young branches were marked using thin strings and a soft bristle brush was used to inoculate the urediniospores on leaves. After inoculation, plants were kept for 24h in a mist irrigation chamber at 25±2°C in the dark, and then they were transferred to a growth chamber at 22±2°C with a 12-h light cycle (80.0 µmol.m-2.s-1) (Ruiz et al. 1989). Approximately 12-20 days after inoculation the plants were evaluated by presence (1) or not (0) of pustules.

DNA extraction

Genomic DNA was extracted directly from a single *P. psidii* pustule (fungus + host tissue) using a modified CTAB-based protocol (Doyle and Doyle, 1987). A single uredinial pustule (>0.5 mm of diameter) was placed in a 2-mL tube containing 100 µL CTAB buffer (2.0% of CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM of Tris-HCl, pH=8.0, and 1.0% of polyvinylpyrolidone). Two metal beads (3.0 mm) were placed in each vial, followed by maceration using a TissueLyser II (Qiagen) for 2 min at 25 rpm. After maceration, the vials were incubated at 5ºC for 5 min. An additional 600 µL CTAB buffer plus 2-mercaptoethanol (2 µL 2-mercaptoethanol to each ml of CTAB buffer) followed by another round of maceration. The vials were incubated in a water bath at 65 ºC for 1 h and mixed by inversion at least five times during the incubation. The samples were centrifuged for 10 min at 13200 g. The supernatant (± 500 µL) was transferred to a new 2-mL tube and 340 µL of isopropanol was added and the mixture gently mixed. The samples were then incubated overnight. The next day the solution was centrifuged at 7000 g for 5 min, and the supernatant was removed gently to avoid pellet disturbance and discarded. Subsequently, 1 mL of 70% ethanol was added to rinse the pellet for 1 min, and then it was centrifuged at 9000g for 1 min. The supernatant was discarded and another 1 mL of 70% ethanol was added to the tube, which was maintained in a shaker at room temperature for 8 hours. After the ethanol was removed, the tube was put upside down over clean towel paper and the pellet was air dried for 30 min. The pellet was eluted in 30 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA) and RNase (2 µg) and the samples incubated at 37°C for 1 hour.

Microsatellite genotyping

The samples were genotyped at six microsatellite loci (EF523503, EF523504, EF523507, EF523508, EF523510, EF523513) (Zhong et al., 2008, Graça et al., 2013). For each 10 µL PCR reaction was used 5 µL of 2x Master Mix (Type-It Microsatellite PCR kit, Qiagen), 0.1 µL (20 µM) of forward (labelled with either D2, D3 or D4 Well-RED fluorescent dye, Sigma-Aldrich) and reverse primers, 0.2 mg/mL of Bovine Serum Albumin (BSA, Fisher BioReagents™), 3.6 µL of nuclease-free water and 1 µL genomic DNA. PCR amplifications were performed using a thermal cycler (model 2720, Applied Biosystems) and the following program: 95ºC for 3 min, then 34 cycles of 94°C for 15s, 45 to 50°C (depending on the locus) for 15s, 72°C for 45s, ending with a hold at 60 °C for 30 min, then 14°C. Fragment analysis was conducted on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter), using 1 µL of PCR product mixed with 38.5 µL Sample Loading Solution (Beckman Coulter) and 0.5 µL size marker (DNA Size Standard Kit – 400, Beckman Coulter).

Analysis

Genetic diversity and population analysis

Isolates were grouped according to the original host that were collected, except for "diverse-host" wherein 12 isolates collected on 12 different hosts were grouped together. Among 88 isolates studied, 13 isolates (ASMG1, CCMG1, CPMG1, ELMG1, LMMG1, PDMG1, SHMG1, SSMG1, SAMG1, SCMG1, SJMG7, EUMG7, PTMG9) were collected in the same location side by side, where a collection of several species of Myrtaceae is maintained. GenAlEx 6.4 (Peakall & Smouse 2006) was used to estimate number of multilocus genotypes, average number of alleles per locus, average number of effective alleles per locus, number of private alleles, Shannon Index of allelic diversity, observed heterozygosity, expected heterozygosity and Fixation Index. The correlation between genetic distance and geographical location were analysed by Mantel test in GenAlEx 6.4 (Peakall & Smouse 2006).

The distribution of genetic diversity was evaluated using the analysis of molecular variance (AMOVA) to partition the genetic variance within and among populations in GenAlEx 6.4 (Peakall & Smouse 2006). A value of Φpt, an analogue of *Fst*, was then calculated as an estimate of differentiation between populations and tested based on 999 random permutations.

To determine if the occurrence of identical multilocus genotype (MLG) was the result of independent sexual reproductive events or clonal reproduction, the p_{sex} approach of Parks & Werth (1993) was performed in MLGsim2.0 (http://www.rug.nl/research/theoreticalbiology/downloads). For that, the following parameters were chosen: MLG for frequency, wherein allele frequencies are based on a subset of the data with every MLG only included once. For model, FIS was used, because this parameter gives a more conservative P_{sex} estimation, taking into account outputs from Hardy-Weinberg equilibrium in the population (Arnaud-Haond et al. 2007). Ten thousand p_{sex} values were simulated, and this distribution was used to estimate a P-value for each MLG in each population.

Principal coordinates analysis (PCoA) based on a covariance matrix with data standardization (Smouse and Peakall 1999) was performed by GenAlEx 6.4 (Peakall and Smouse 2006). The presence of genetic clusters in the dataset was analysed by the model-based Bayesian method implemented in STRUCTURE v2.3.4 (Pritchardet al. 2000). For STRUCTURE analyses, an admixture model was assumed, correlated allele frequencies and geographic location of sampling was not used in clustering. The Monte Carlo Markov Chain (MCMC) sampling scheme was run for 500,000 iterations with a 100,000 burn-in period, with K ranging from 1 to 12 and 10 independent replications for each K. The optimal value of K was inferred using the method of Evanno et al. (2005) implemented in STRUCTURE HARVESTER (Earl & von Holdt 2012) available on http://taylor0.biology.ucla.edu/structureHarvester/. Both PCoA and STRUCTURE analyses were performed using all isolates (n=88).

Analysis of physiological groups and correlation between genetic and physiological variability

As the majority of the isolates were collected in wild plants, only 50 of the 88 isolates had enough spores to inoculate the host set. For each isolate, the results of the inoculation in each host was scored as 1 (presence of pustules) and 0 (absence of pustules). The isolates were classified into physiological groups based on the same results on the host set. The binary results from each group were used to construct the Euclidean Distances of isolates calculated by hierarchical cluster analysis. All 50 isolates were used to calculate the correlations between either geographic distance of the isolates or genetic distance and the physiological group distance evaluated by the Mantel test using GenAlEx 6.4 (Peakall & Smouse 2006) with 1,000 permutations.

The genetic and physiological group dendrograms were constructed by unweighted pair group method with arithmetic mean (UPGMA) and euclidean distance, respectively.

Results

Genetic diversity and population structure

Eighty eight isolates of *Puccinia psidii* collected in 20 host species in five Brazilian states were scored using six microsatellites. Considering all isolates, 2 to 11 alleles were detected per locus; the loci 508 and 510 were the least and most polymorphic, respectively (Table 1). Within populations, the average number of alleles per locus ranged from 1.667 in the *E. florida* population to 4.833 in the diverse-host population, respectively (Table 2). The effective number of alleles ranged from 1.595 in the *E. florida* population to 2.984 in isolates collected from *M. cauliflora*. There were no private alleles for *P. guineense*, *Eucalyptus* spp. and *S.cumini* populations, whereas five private alleles were observed in the diverse-host population (Table 2). Shannon information index of allelic diversity varied from 0.4 to 1.15 for *E. florida* and diverse-host population, respectively (Table 2). For all populations the observed heterozygosity was higher than the expected heterozygosity (Table 2).

All populations showed a high negative fixation index (F_{IS}= -0.179 to -1) indicating an excess of heterozygotes. Forty eight multilocus genotypes (MLG) were identified (Table 3), of which 32 MLGs were unique. Only the *S. cumini* population showed a single MLG. For all isolates, the MLGs were designated according to the original host, except four isolates (SSMG1, SAMG1, EUGENIA-MH, SHMG1) grouped within diverse-host population that shared two MLGs with *Eucalyptus* spp. and one with *P. guineense* populations (Table 3).

The Mantel correlation coefficient using the genetic distance matrix and geographic distance matrix for all isolates were not significant (p> 0.05 and r= -0.069). For 13 isolates (ASMG1, CCMG1, CPMG1, ELMG1, LMMG1, PDMG1,

SHMG1, SSMG1, SAMG1, SCMG1, SJMG7, EUMG7, PTMG9) collected from different hosts side by side in the same location, 12 MLGs were found.

For AMOVA results, based on Φpt, all isolates (n=88) were considered and 42% of the molecular variance occurred within individuals and 58% of the variance was due to differences among populations, or host of origin ($P < 0.001$). The same test was rerun excluding the isolates grouped in diverse-host (n=76) and the molecular variance within individuals and among populations was 35% and 65%, respectively ($P < 0.001$). The null hypothesis that identical MLG was due to independent sexual reproductive events was rejected ($P<0.001$) for 11 of 16 MLGs (Table 4). For the remaining five MLGs the null hypothesis was not rejected ($P> 0.05$), four of them are from *Eucalyptus* spp.

The STRUCTURE analyses indicated K=2 as the best number of genetic clusters using the Evanno et al. (2005) method. The entire sample set were classified in two different groups; one of which (red) contained isolates collected from *Eucalyptus* spp., *E. florida*, *P. guineense*, and *S. jambos*, while the other group (green) contained isolates collected from *P. guajava*, *M. cauliflora*, *S. cumini*, and *E. uniflora* (Figure 2). The isolates from the diverse-host population were dispersed into both groups, with seven isolates clustering with the eucalyptus group and two with the guava isolates. A transition group between the two clusters was also observed and included one isolate from *P. guineense*, three from *S. jambos*, three from *P. guajava* and three from the diverse-host population.

According to PCoA results there is a genetic differentiation among genotypes derived from different hosts (Figure 3). Different clusters formed by genotypes associated with *E. uniflora*, *M. cauliflora*, *S. cumini*, *E. florida* and *Eucalyptus* spp. were observed. Genotypes associated with *P. guajava* formed two distinct and distant clusters. Genotypes associated with *S. jambos*, *P. guineense* and the diverse-host population were not grouped in a specific cluster. The axes 1 and 2 explained 30% and 17%, of the genetic variation, respectively.

Physiological groups and correlation between genetic and physiological variability

All isolates infected the original host species. Isolates from *P. guineense* infected *P. guajava* and *S. jambos*, isolates from *E. florida*, *Eucalyptus* spp., *S. cumini* and *M. cauliflora* also infected *S. jambos*. All isolates from *P. guajava*, *S. cumini* and *E. uniflora* infected *P.guineense*. All isolates from *S. cumini* and diverse-host population also infected *M. cauliflora* and isolates collected on *S. jambos* infected eucalyptus. The remaining results varied according to isolate-host tested. Only two isolates, JBMG5 and ASMG1 were able to infect *E. uniflora* (Figure 4, Table 5).

Among 50 isolates tested, 17 physiological groups were identified (Table 5), group 9 was the most prevalent (Figure 4). Although the correlation was very small, the Mantel tests ($p< 0.01$, $r= 0.061$) showed an association between the Euclidean distances of the physiological groups and genetic distance matrices. The correlation between Euclidean distances of physiological group matrix and corresponding geographic location matrix was not significant ($p> 0.05$, $r= -0.178$). Each physiological group contained multiple genotypes. For example, group 5 comprised seven isolates and each has a different MLG. Similarly isolates with the same MLG shared different physiological groups. Isolates EUSP1A, EUSP2 and EUSP4 (all of them MLG 20) were assigned in three different physiological groups, 10, 5 and 9, respectively. There was no complete match between physiological groups and genotype.

Discussion

In a single study, cross-inoculations and microsatellite marker analysis were combined to investigate the correlation between genotype and physiological groups of the myrtle rust population. Selection of genotypes by host species regardless location and other features of the genetic structure of the 88 isolates collected on 20 host species were similar to the previously reported by Graça et al. (2013). Both, the strength of selection by host and the lack of influence of geographic location on genetic differentiation among isolates were observed mainly in a collection of species of Myrtaceae naturally infected by rust. Among 13 isolates collected side by side on different hosts, 12 MGLs were found. However, the isolates SCMG1, SJMG7, EUMG7, PTMG9, collected respectively on *S. cumini*, *S. jambos*, *Eucalyptus* spp., and *Eugenia uniflora*, shared the same MLGs with other isolates collected on their respective hosts in different locations.

The results of MLGsim and the high negative values of the fixation index (FIS) suggest that *P. psidii* population is mainly clonal, as described by Graça et al. (2013). Under clonal reproduction, FIS is expected to be negative, indicating an excess of heterozygotes relative to random mating (Halkett et al. 2005). In contrast with previously studies (Graça et al. 2013) the genotypes of *P. guineense* were clustered by PCoA and STRUCTURE together with genotypes of *Eucalyptus* spp. rather than genotypes from *P. guajava*. Besides that, the Shannon information index of allelic diversity for *Eucalyptus* spp. and *P. guajava* isolales were more diverse in this study.

Cross-inoculations showed physiologic variation among isolates collected on different hosts. But in general these results are similar to Castro et al. (1983), Coelho et al. (2001), Aparecido et al. (2003b), but distinct from Ferreira (1981). Some issues should be considered for this study wherein the majority of isolates were collected on wild plants. For example, most inoculations were done once due to low amount of spores of each isolate. When possible, inoculations were performed on the original host species. However, in several cases the difficulties to obtain and multiply the original plant material made impossible inoculations onthe original host species. Thus, *S. jambos* or *P. guineense*, the two most susceptible species, served as positive controls indicating that inoculations were successful. Except for *Eucalyptus urophylla*, for the other hosts seedlings were used because a set of clonal plantsis not available for all species yet.

When analyzed separately, microsatellite markers and cross inoculations have indicated a specialization according to the host species. Although the association between physiological groups and genotype matrices was significant, the correlation between them is very small. Besides the genetic background of the isolate and host, other causes can influence the result of the inoculation such as period of storage of the spores (Salustiano et al. 2008) and phenology stage of the host.

The low or no correlation between physiological groups and molecular analyses has been found in other rust species as *Puccinia triticina* (Dickinson et al. 1990, Kolmer 2001), *P. striiformis* f. sp. *tritici* (Chen et al. 1993), and *Puccinia coronata* f. sp. *avenae* (Brake et al.2001) and *P. graminis* f. sp. *avenae* isolates collect in Australia (Keiper et al., 2006). According to Chen et al. (1993) the DNA polimorfism is independent of virulence once the genome evolves faster than genes that control virulence, which are under host selection pressure.

Although *P. psidii* population is mostly clonal there is a high genetic and physiological variability within and among fungal populations. Among 88 isolates there were 48 MLG, 32 of them were unique. For the cross-inoculation tests the entire set sample (n=50) were grouped in 17 groups, 8 of them were designed an unique genotype.

Group 9 was the most common physiological group and it was found 19 times. The same was observed for *Puccinia striiformii* f sp. *tritici* in China, although the pathogen is strictly asexual there is a high level of genetic diversity (Shan et al., 1998) and diversity of virulence patterns (Wan et al., 2004).

The mechanism by which *P. psidii* alters its host range is unknown. Recombination is one of the mechanisms to induce genetic variation that may result in an altered host range, but its real contribution is unknown. Moreover the lifecycle of the pathogen is still unclear. Teliospores of *P. psidii* have been observed in the field (Alfenas et al. 2009, Perez et al, 2011, Pegg et al. 2013) and artificial inoculation producing basidiospores under ideal environment conditions (Ruiz et al, 1989, Aparecido et al, 2003a). However, there is no conclusive evidence on the infectivity of basidiospores and what their biological role in the *P. psidii* lifecycle (Morin et al.2014, Machado et al. 2015).

Mutation accompanied by strong selection pressure exerted by the host species in *P. psidii* may generate high variability of genotype and physiological groups as proposed for *Puccinia triticina* from wheat (Kolmer, 2001). In a study of races of *P. striiformis* f. sp. *tritici* in China, the authors hypothesized that the base of high number of race was due to mutation (Chen et al., 2009), however other author (Mboup et al 2008) demonstrated also a correlation between somatic recombination, detected by microsatellite analysis with changes in the spectrum of virulence.

The existence of multiple physiological groups with the same microsatellite genotype would indicate that mutation rather than recombination is the basis for variation in the host range of *P. psidii* population. Transposable elements (TEs) may also play a role in the variability of *P. psidii* once they can determine genetically heritable variation independently and additionally to sexual recombination (Biémont and Vieira, 2006). Transposable elements represent almost 50% of the genome of rust species as *P. graminis* f. sp. *tritici*, *Melampsora larici-populina* (Duplessis et al, 2011) and 25% for *P. psidii* according to a brief overview of the composition genome (Tan et al. 2014). If TEs, mutation or other mechanisms are involved in the host range of *P. psidii* will be better understood by a comparative genome of isolates of the pathogen collected in different host species.

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Table 1 – Six microsatellite primers used to genotype *Puccinia psidii* isolates collected from 20 Myrtaceae host species in Brazil

Locus	GenBank Accession	Primer Sequence (5' -3')	Repeat motif	No. Alleles
PpSSR0	EF523503	F: TCTTGGTTATCGAGCCTGGT R: TTGAGTAGTCCGAATTCGCC	$(TC)_9$	5
PpSSR0	EF523504	F: CCTTTAGGCTGTGGTTTCCA R: TTCCTCTTGACAGAGTGGGC	$(TC)_{12}$	6
PpSSR0	EF523507	F: AAGAACGTGAACGGGAATGA R: TACCCTTCGTCTGGCATTTC	$(AG)_{14}$	10
PpSSR1	EF523508	F: TGACTTTAATCATCTTCAAAACCAA R: GATGAAGGAAGGGGATTGGT	T_7 + $(AG)_{22}$	2
PpSSR1	EF523510	F: TTGGTAAAGAGGAGGGGATTC R: GAAGGTAATGGTTGGTGCTGA	$(AG)_{73}$	11
PpSSR1	EF523513	F: GAACGAACCCAAACTTTCCA R:TGTGTTCAATCTCATTCCTTTCC	$(TC)_{18}$	6

Table 2- Allelic and genotypic variation in isolates of *Puccinia psidii* from different hosts

Hosts	Sample Size	N of multilocus genotype	Allele/ locus	Efective allele/ locus	Private alleles	Shannon index	Ho[a]	He[b]	Fixation index
Psidium guineense	3	3	2.16	2.03	0	0.62	0.56	0.39	-0.42
Eugenia florida	3	3	1.66	1.59	2	0.4	0.5	0.27	-0.88
Eucalyptus spp	30	10	2	1.90	0	0.62	0.61	0.42	-0.42
Myrciaria cauliflora	11	9	4	2.98	2	1.05	0.73	0.55	-0.33
Psidium guajava	13	7	2.5	1.89	1	0.61	0.46	0.36	-0.18
Eugenia unifora	5	3	2	1.94	1	0.68	0.9	0.48	-0.85
Syzygium cumini	3	1	1.83	1.83	0	0.58	0.83	0.42	-1
Syzygium jambos	8	4	2.5	2.04	1	0.72	0.81	0.46	-0.79
Diverse-host	12	11	4.83	2.89	5	1.15	0.72	0.60	-0.21

Ho= Observed heterozygosity
He= Expected heterozygosity

Table 3 – Isolates, host, State of origin and multilocus genotype (MLG) of *Puccinia psidii* isolates collected in Brazil. In bold isolates grouped in diverse-host that shared the same MLGs with different populations.

Host	Isolate	State[a]	MLG[b]	Host	Isolate	Estate	MLG[b]
P.guineense	ARES04	ES	48	*M. cauliflora*	JBES2A	ES	4
	ARES3	ES	18		JBES3	ES	35
	SHMG1	MG	18		JBES4A	ES	32
	ARMG4	MG	26		JBES5	ES	9
E. florida	EFMG1	MG	25		JBES7	ES	6
	EFMG2B	MG	24		JBMG4	MG	6
	EFMG3	MG	10		JBMG08	MG	3
Eucalyptus spp.	EUBA1	BA	21		JBMG2	MG	44
	SSMG1	MG	21		JBMG5	MG	2
	UFV2	SP	21		JBES6	ES	2
	SAMG1	MG	21		JBSP1A	SP	1
	EUES10A	ES	27	*P.guajava*	PGES1A	ES	33
	EUES25	ES	27		PGES3A	ES	34
	EUES22A	ES	28		PGES4A	ES	22
	EUES20A	ES	28		PGMG14	MG	36
	EUSP7A	ES	28		PGMG15	MG	7
	EUSP5	ES	28		PGMG20	MG	37
	EUES26A	ES	29		PGMG17A	MG	37
	EUES19A	ES	29		PGMG4A	MG	37
	EUES27A	ES	12		PGMG6	MG	37
	EUES21A	ES	12		PGMG19	MG	37
	EUES28A	ES	30		PGMG16A	MG	37
	EUES12A	ES	30		PGMG3B	MG	37
	EUES3	ES	23		PGSC10	SC	38
	EUES4A	ES	31	*E. uniflora*	PTMG08	MG	40
	EUES5A	ES	31		PTMG7A	MG	39
	EUES6	ES	31		PTMG6	MG	39
	EUES7A	ES	31		PTMG3	MG	39
	EUES1	ES	31		PTMG9	MG	41
	EUGENIA-MG	MG	19	*S.jambos*	SJES2	ES	43
	EUES30A	ES	19		SJMG7	MG	8
	EUES17A	ES	19		SJMG3	MG	16
	EUES11A	ES	19		SJMG2	MG	16
	EUMG07	MG	19		SJMG1	MG	16
	EUSP6	SP	20		SJMG4	MG	16
	EUSP2	SP	20		SJES1B	ES	16
	EUSP4	SP	20		SJSC1	SC	42
	EUSP1A	SP	20	Diverse-host[b]	ASMG1	MG	5
	EUSP3A	SP	20		CCMG1	MG	13
	EUES2	ES	20		CPMG1	MG	11
S. cumini	SCMG1	MG	46		ELMG1	MG	17
	SCSP1A	SP	46		LMMG1	MG	14
	SCMG2A	MG	46		PDMG1	MG	47
					EUGENIA-ES	ES	45
					ESES1	ES	15

[a] ES= Espirito Santo, MG= Minas Gerais, BA= Bahia, SP= Sao Paulo and SC= Santa Catarina

[b] ASMG1= *Acmena smithii*, CCMG1= *Callistemon citrinus*, CPMG1= *Callistemon pachyphyllus*, ELMG1= *Eugenia leitonii*, LMMG1= *Leptospermum madidum*, PDMG1= *Pimenta dioica*, SHMG01= S*yzygium coffs harbour*, SSMG01= *Syzygium saramange*, SAMG01= *Syzygium australe*, EUGENIA-MG and EUGENIA-ES= *Eugenia* sp., ESES1= *Eugenia stipitata.*

Table 4 - Probabilities of repeated sampling of multilocus genotypes arising from sexual reproduction in *Puccinia psidii* populations.

MLG	n	P_{Gen}	P_{Sex}	P-value	Level
2	2	1.05E-08	4.23E-13	0.0000	***
6	2	1.96E-08	1.47E-12	0.0000	***
12	2	0.00046091	0.000792035	0.5031	ns
16	5	1.45E-05	1.33E-15	0.0000	***
18	2	0.000117282	5.23E-05	0.2112	ns
19	5	0.000553092	1.95E-09	0.0000	***
20	6	0.000268407	1.96E-13	0.0000	***
21	4	0.000299235	1.83E-08	0.0000	***
27	2	0.000419009	0.000656145	0.5031	ns
28	4	0.000203339	3.93E-09	0.0000	***
29	2	0.000226693	0.000194181	0.3789	ns
30	2	0.000502811	0.000940333	0.5217	ns
31	5	0.000244006	3.33E-11	0.0000	***
37	7	2.94E-06	2.89E-15	0.0000	***
39	3	2.16E-10	0	0.0000	***
46	3	1.95E-09	0	0.0000	***

*** $P < 0.001$, ns= no significant ($P> 0.05$)

Table 5 –Physiological groups observed after inoculation of 56 isolates of *Puccinia psidii* on six Myrtaceae species.

	Inoculated host species					
Physiological group	***Eucalyptus urophylla* (clone CLR 079)**	***Psidium guineense***	***Psidium guajava***	***Syzygium jambos***	***Myrciaria cauliflora***	***Eugenia uniflora***
1	1	1	1	1	1	1
2	0	1	1	1	1	0
3	0	1	1	0	0	0
4	0	1	1	0	1	0
5	1	1	1	1	1	0
6	1	1	1	1	0	0
7	1	0	1	1	1	0
8	1	1	0	1	1	1
9	1	1	0	1	1	0
10	1	1	0	1	0	0
11	0	0	0	1	1	0
12	1	1	0	0	1	1
13	0	0	0	1	0	0
14	0	1	0	1	1	1
15	0	1	0	1	1	0
16	0	0	1	1	1	0
17	1	1	1	0	0	0

0 = Absence of pustules
1 = Presence of pustules

Figure 1 - Sampling locations of *Puccinia psidii* in five states in Brazil

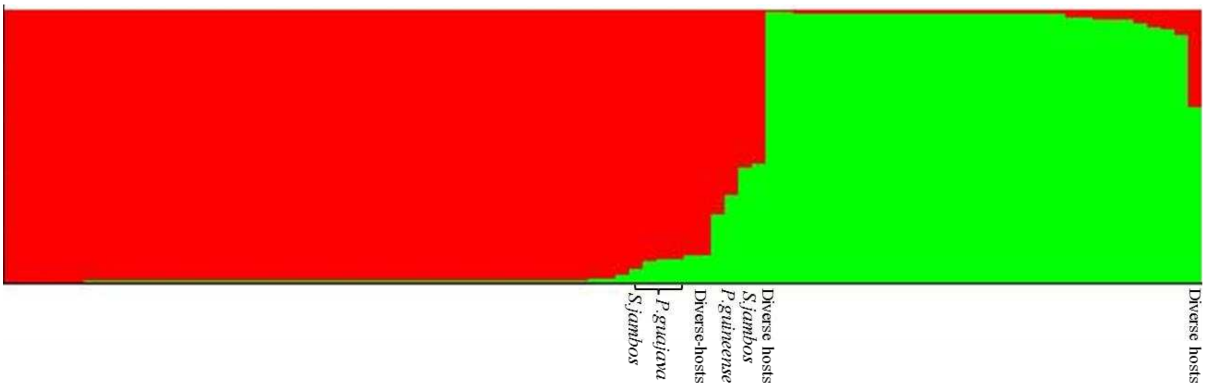

Figure 2 - Genetic structure of *Puccinia psidii* population analysed by the model-based Bayesian method implemented in STRUCTURE v2.3.4. In red isolates collected from *Eucalyptus* spp., *E. florida*, *P. guineense*, and *S. jambos*. In green isolates collected from *P. guajava*, *M. cauliflora*, *S. cumini*, and *E. uniflora*

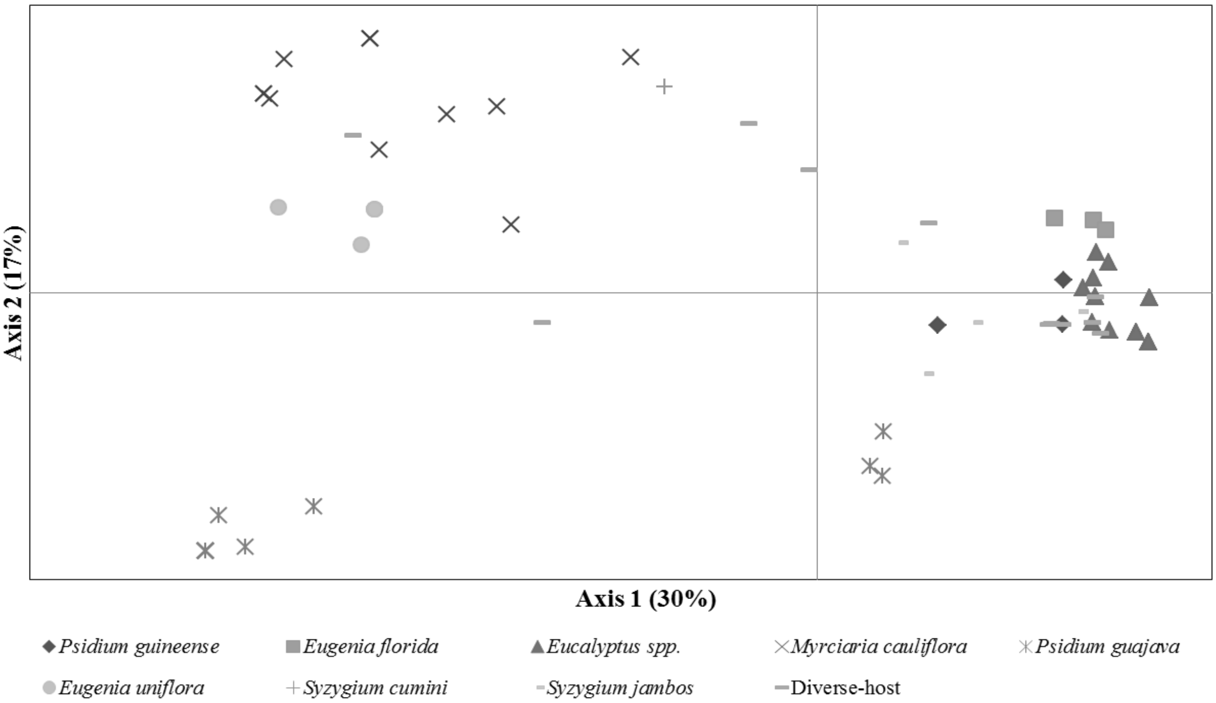

Figure 3 - Principal coordinates analysis of multilocus genotypes observed to 88 isolates of *Puccinia psidii*. Coordinates are based on a covariance matrix with data standardization and the first two axes explain 47% of the observed variation.

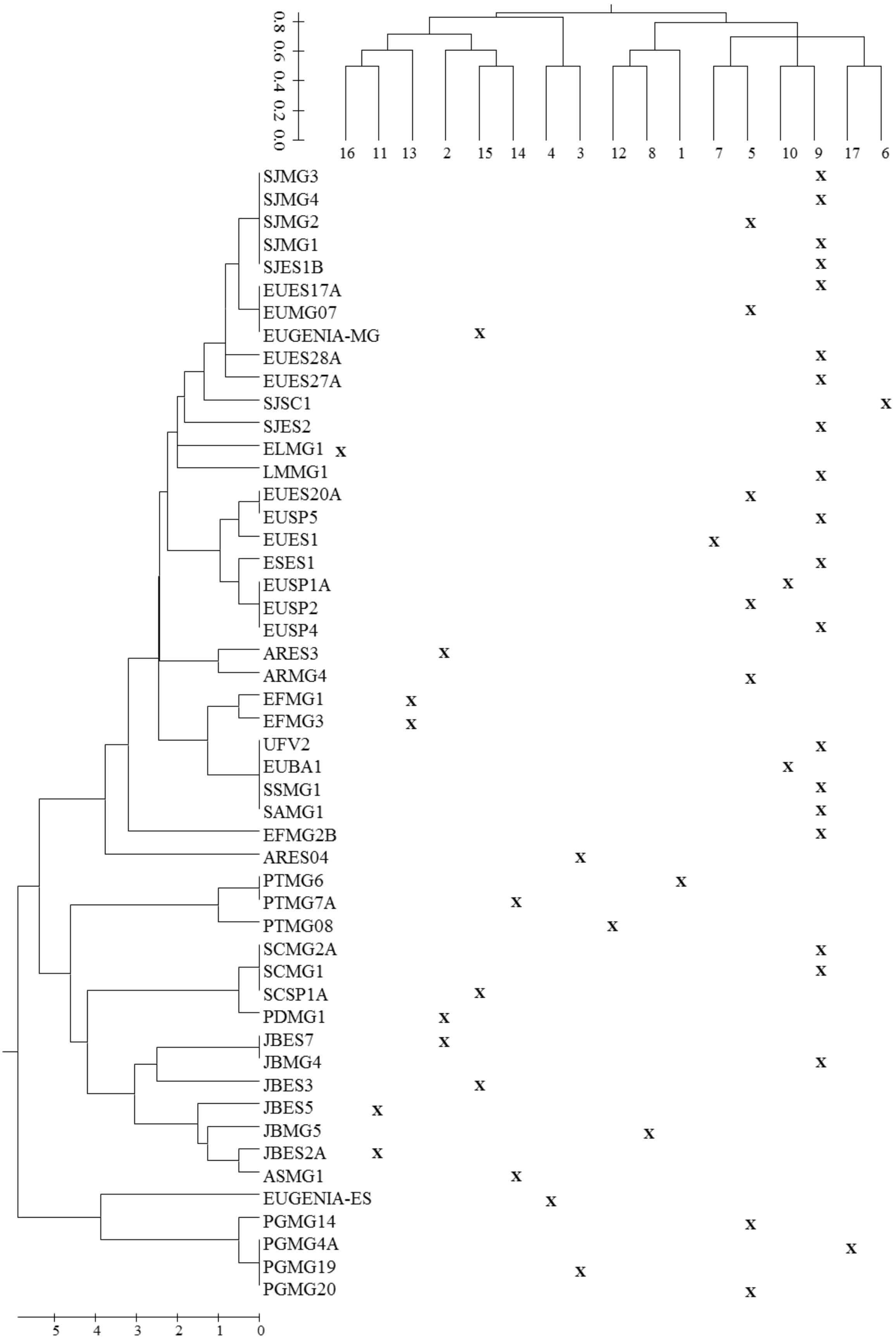

Figure 4 - Distribution of isolates of *Puccinia psidii* analysed according to their physiological groups. Genotype tree (left) and physiological group tree (top) were clustered by UPGMA.

ARTICLE 5

Genomic analysis of *Puccinia psidii* using Next Generation Sequencing

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Abstract

The aim of this study was to provide the genome sequencing of five isolates of *Puccinia psidii* from Brazil and Australia and a brief genomic analysis among them. The assembly of each isolate was compared to VIC 42496. Analysis reveled that more than 70% of the contigs of each isolate that was no present on assembly of VIC42496 had no significant homology (no hits) to anything currently residing in the fungal genome databases. Contigs of each isolate with matching sequences in the NCBI database had highest similarity to bacteria and other organisms such as insects and plants. For those that had similarity to fungal sequences, including *P. psidii*, the majority were homologous to genes coding for cellular components, biological, molecular process or hypothetical proteins. Only three contigs matched to genes that code for products that could be involved with infectivity. The access to all these genomes will facilitate further research to development efficient strategies to control rust disease in agricultural and forest ecosystems.

Keywords: Myrtle rust, DNA assembly, next generation sequencing

Introduction

Puccinia psidii was first reported in 1884 (Winter) on guava plants (*Psidium guajava*) in São Francisco do Sul/Brazil, and since then it has spread from South to North America (Maclachlan, 1938, Marlatt and Kimbrough 1979, Coutinho et al., 1998, Mellano, 2006, Uchida et al., 2006). In recent years, *P. psidii* has migrated to other continents, including Asia (Kawanishi et al., 2009, Zhuang and Wei, 2011), Africa (Roux et al., 2013), and Oceania (Carnegie et al., 2010, Giblin, 2013). The symptoms depend on the susceptibility of the host, but can range from small pustules with few spores to massive production of powdery bright yellow urediniospores with shoot dieback that may ultimately result in plant death (Alfenas et al., 2009).

Molecular studies of *P. psidii* have started a few years ago with population geneticstudies of the pathogen in Hawai'i (Zhong et al., 2011) and Brazil (Graça et al., 2013). These studies indicate that *P. psidii* has reproduced clonally in Hawai'i and that populations from different hosts in Brazil are derived from different lineages. More recently, a brief overview of the complete genome of *P. psidii* strain present in Australia estimated its genome size to be between 103 and 145 Mb with more than 19,000 genes (Tan et al., 2014).

Next generation sequencing (NGS) is a powerful tool that can provide high quality whole genomic sequencing much more rapidly and cheaply than previous sequencing technologies (Cantu et al., 2011). Whole genome sequencing has already been applied in plant pathology and in plant-microbe interaction research to infer the evolution of pathogens and the factors of pathogenicity and virulence genes (Cantu et al., 2011), comparative analyses between different races or species (Duplessis et al., 2011) and identification of effector proteins (Joly et al., 2010). This technology has been used in studies of several rust species including *Puccinia striiformis* Westend f.

sp. *tritici* Eriks (Cantu et al., 2011), *Puccinia graminis* f. sp. *tritici* (Duplessis et al., 2011) and *Puccinia triticina* (http://www.broadinstitute.org/annotation/genome/puccinia_group/Info.html), stripe, stem and leaf wheat rust, respectively, *Melampsora larici populina*, poplar leaf rust (Duplessis et al., 2011) and *Melampsora lini*, flax rust (Nemri et al., 2014).

Understanding mechanisms of pathology and general biology of rust fungi and devising innovative ways to protect crops against them can be facilitated by genome sequencing. The aim of this study was to produce a draft genome sequence for five isolates collected on differen hosts to be used as reference of *P. psidii*. Such studies will provide information on the biology of the pathogen such as genomic basis of its selective pathogenicity on different hosts, which may enable the development of rapid screening methods for discrimination of the different *P. psidii* biotypes.

Material and methods

Sampling, multiplication of spores and genomic sequencing

Four isolates of *P. psidii* from Brazil: ARES4 (*Psidium guineense* SW) EGUFV2 (*Eucalyptus grandis* Hill ex Maiden), PTMG8 (*Eugenia uniflora* Berg), VIC42496 (*Psidium guajava* L), and one isolate GP (*Rhodamnia sessiliflora* Benth.) from Australia (Table 1) were sequenced. Each isolate was geo-referenced and spores were collected separately in 1.5-mL Eppendorf® tubes. The spores of each isolate were multiplied on plants of the original host species. Seedlings were maintained in 2-L plastic bags, containing the substrate MecPlant® (partially decomposed pine bark and vermiculite), supplemented with 3 Kg/m^3 of Osmocote® and 8 Kg/m^3 of simple super phosphate. For each plant, 4-5 healthy young branches were marked with thin strings and a soft bristle brush was used to inoculate the urediniospores on leaves. After inoculation, plants were kept for 24 h in a mist irrigation chamber at 25±2°C in the dark, and then they were transferred to a growth chamber at 22 ±2°C with a 12 h light cycle (80 μmol m^{-2} s^{-1}) (Ruiz et al. 1989). Approximately 12-20 days after inoculation, urediniospores were collected by brushing onto aluminium foil using an autoclaved soft bristle brush and stored at -80°C prior to DNA extraction.

DNA extraction and genome sequencing

DNA was extracted using a Qiagen DNeasy Plant minikit. Two tungsten carbide beads were added to each 1.5 mL microcentrifuge tube containing approximately 100 μL of spores. Spores were disrupted in a Qiagen Tissuelyser for 1 min at 30 Hz frequency and the DNA was extracted according to the kit instructions, except that the 65°C incubation step was extended from 10 min to 1 h. DNA was eluted in TE buffer.

Libraries were prepared from bead size-selected DNA fragments from each sample of genomic DNA and sequenced on an Illumina HiSeq according to the manufacturer’s instructions at the Australian Genomic Research Facility. A higher coverage was requested for VIC42496 to allow this to be used as the reference sequence for comparative analyses.

Contig analysis

For each isolate the paired-end sequences were trimmed to remove low-quality sequences using the CLC Genomics Workbench v7.5 (CLC bio, Aarhus, Denmark). A limit value of 0.05 was used for the quality trimming with a maximum number of two ambiguous nucleotides at the sequence ends allowed. Reads were assembled into a draft genome using CLC-Bio Genomics Workbench v7.5 (CLC bio, Aarhus, Denmark) to obtain the highest N50 value and the longest contigs. The following parameters were applied for the CLC-Bio Genomics Workbench: mismatch, insert and deletion cost = 3; length fraction = 0.5; similarity fraction = 0.75; minimum contig length = 1000; bubble size = 250. After the initial assembly, reads were mapped back to contigs and contigs updated.

Comparative genomic analysis

The assembly of each isolates were compared with VIC42496 using blastn (Figure 1), as this isolate has the highest sequencing coverage. Exclusive contigs (no-hits) of each isolate were obtained and matched separately by blastn against the nucleotides database of NCBI (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) using a cutoff expected (*E*) value of 1E–10 to identify known sequences or genes.

Results

Total of reads, trimmed and discarded reads, numbers of contigs, and other basic statistics for each assembly are summarized in Table 2. The number of reads ranged according to the sequencing coverage of each isolate. The reads of each isolate were trimmed to remove low-quality which reduced the average length from 150 bp to 147 bp. For all isolates the estimated GC content was 33%.

Using blastn, the comparative genomic analysis of assemblies of ARES4, EGUFV2, GP and PTMG8 against VIC42496 revealed, respectively, 1184, 1914, 844 and 841 contigs that were not matched with any contig from VIC42496. Among those contigs of ARES4, EGUFV2, GP and PTMG8, 1117, 1375, 817 and 843, respectively, had no significant homology (no hits) to anything currently residing in the fungal genome databases. The majority of contigs with matching sequences in the NCBI database had highest similarity to bacteria and other organisms such as insects and plants (Figure 2), except PTMG8 that had 100% of similarity to fungal sequences. All of them matched with *P. psidii*. For the isolates ARES4, EGUFV2 and GP, only four, four and three contigs, respectively, matched sequences from *P. psidii* (Table 3).

Discussion

For the comparative genomic analysis, the most part of contigs of ARES4, EGUFV2, GP and PTMG8 that were not present in VIC42496 had similarity mainly to bacteria. Even DNA extracted from organisms that can be isolated in culture may have contamination from other organisms and this is particularly hard to avoid for obligate biotrophs like rust fungi. Due to the massive sequences of bacteria deposited on GenBank is also common small and conserved sequences match with any bacteria sequences.

Of the contigs that found matching fungal sequences, including *P. psidii,* the majority were homologous to genes coding for cellular components, biological, molecular process or hypothetical proteins. However, EGUFV2 and PTMG8 had one contig homologous to a pectate lyase gene from *P. psidii* (KF431996). Additionally, a contig from EGUFV2 matched the AMT gene region from *Alternaria alternata* (AB525199 and AB525198) (Table 3). Both of these genes code for products that could be involved with infectivity. Pectate lyases degrade the pectate component of the plant cell wall and are widely distributed in diverse families of plants and microorganisms including bacteria such as *Erwinia carotovora, Pseudomonas* and *Xanthomonas* and fungi such as *Aspergillus, Fusarium,* and *Penicillium* (Dubey et al., 2010). The AMT genes it is related to AM-toxin biosynthesis, a host-specific toxin produced by *Alternaria alternata* to infect susceptible apple cultivars (Johnson et al., 2000). Gene sequences with a possible involvement in infectivity were not found among the exclusive contigs of the other isolates.

It is unknown if the contigs that had no significant similarity to any sequence currently available on GenBank database are derived from the *P. psidii* genome or from contaminant organisms. Although the genome sequencing of different rusts has revealed conserved regions among species (Stukenbrock et al., 2010), many genome regions are considered species-specific or even race-specific this fact could be partially responsible for the lack of detection those sequences (Stukenbrock et al., 2010, Cantu et al. 2011)

The genome size of *P. psidii* estimated to a Australian isolate ranged from 103 to 154 Mb (Tan et al. 2014), comparable to other rust species such as *P. triticina* (100–120 Mb) and *P. striiformis* (110 Mb) (www.broadinstitute.org) and *Melampsora larici-populina* (101 Mb; Duplessis et al. 2011). Up to now we have done a draft genome sequence of five isolates of myrtle rust and rapid and simple analyses of the differences among them, but the estimated size of genome was not estimated due to the complexity of the data. A study using flow cytometry is ongoing to estimate it.

As shown on the workflow (Figure 1), it is possible to analyse the matches (hits) among the isolates. This strategy will allow us to find fine differences such as SNPs, identify candidate genes involved in host specialization and specific effectors. Rust genes encoding effectors can exhibit high levels of polymorphism and signatures of positive selection (Joly et al., 2010). Thus, identifying the effectors is possible to infer their function and the evolutionary processes involved on host range of rust species (Stukenbrock et al. 2010)

Cross-inoculation (Castro et al., 1983, Coutinho and Figueiredo, 1984, Ferreira, 1981, Coelho et al., 2001, Xavier 2002, Aparecido et al., 2003) and microsatellites analyses (Graça et al. 2013) indicate a specialization of *P. psidii* according to the host but the mechanisms by which the pathogen alters its host range is unknown. As performed for *Mycosphaerella graminicola* (Stukenbrock et al. 2010), using comparative genome analysis of *P. psidii* isolates it might be possible to infer how speciation and host specialization processes have influenced pathogen evolution. The ability to infect particular plant species could be related to specific genes which encode host-determining virulence factors including small secreted proteins (SSPs) and enzymes involved in the synthesis of toxins (Van der Does and Rep, 2007). Most SSPs that are specifically produced during plant infection are likely to be effectors that manipulate host cells to facilitate parasitic colonization, such as by suppressing plant innate immunity or enhancing nutrient availability (Ellis et al., 2009). In silico gene prediction and manual annotation of SSP genes in *M. larici-populina* and *P. graminis* f. sp. *tritici* genome identified, respectively, a set of 74% and 84% of SSPs that are lineage-specific (Duplesis et al 2011). For *Fusarium oxysporum* and *Ustilago maydis*, it was also identified genes that are specific for the species or even forma speciales (Kamper et al., 2006, van der Does and Rep, 2007). Due to the low conservation among candidate effectors only a small proportion of sequences of *P. striiformis* f. sp. *tritici* had significant similarities to sequences in the GenBank (Cantu et al. 2011). Identifying effector genes in *P. psidii* is therefore expected to be a non-trivial task.

The genome sequencing of five isolates of *P. psidii* provided here opens the opportunity to track the genetic determinants for successful infection on different hosts. The isolates selected for this study are particularly representative as EGUFV2, race 1, is the most common on eucalyptus in Brazil. VIC42496, collected on guava, is the epitype of *P. psidii* (Machado et al. 2015), ARES4 was collected on *P. guineense* a close species to *Psidium guajava*, PTMG8 represent isolates from *E. uniflora* which have a unique profile according cross-inoculation results (Machado, unpublished data) and GP represents an isolate of the strain recently introduced into Australia.

The access to all these genomes may facilitate further research on this important pathogen, including a deeper understanding of pathogenicity, virulence factors, effectors and their evolution affecting host–pathogen interactions. The information thus gained will enhance the development of efficient strategies to control rust disease in agricultural and forest ecosystems, particularly in the development of resistant host material.

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Table 1- *Puccinia psidii* isolates selected for comparative genomic analysis.

Isolate	Host	Location[a]	Country	Coordinates	
ARES4	*Psidium guineense*	Conceição Castelo/ES	Brazil	-20,79	-42,79
EGUFV2	*Eucalyptus grandis*	Itapetininga/SP	Brazil	-22,59	-48,8
GP	*Rhodamnia sessiliflora*	Brisbane/QLD	Australia	-27.30	152.57
PTMG8	*Eugenia uniflora*	Viçosa/MG	Brazil	-20,76	-42,85
VIC42496	*Psidium guajava*	Araquari/SC	Brazil	-26,37	48,71

[a] ES= Espírito Santo, SP=São Paulo, QLD= Queensland, MG=Minas Gerais, SC=Santa Catarina

Table 2 - Basic statistics of genome assemblies for five isolates of *Puccinia psidii*

	VIC42496	ARES4	PTMG8	EGUFV2	GP
Sequence quality					
Total # of reads	202,650,874	33,699,244	32,423,984	35,689,256	31,372,976
Total of trimmed reads	16,918,158	3,905,015	2,688,902	2,854,778	2,556,702
Discarded reads	3,656,073	430,325	551,168	663,456	513,239
Assembly					
Including scaffolded region					
Total # of contigs	303,041	79,559	96,146	102,124	82,324
N50 (bp)	3,668	1,402	1,275	1,373	1,252
Max contig length (bp)	252,049	92,716	49,184	44,637	49,713
Total length (bp) [a]	687,274,516	98,863,463	110,395,153	123,026,400	93,286,023
Excluding scaffolded region					
Total # of contigs	449,632	112,025	118,433	125,231	101,858
N50 (bp)	2,761	1,091	1,129	1,207	1,098
Max contig length(bp)	109,645	29,326	39,608	32,864	22,176
Total length (bp) [a]	677,748,361	95,764,172	109,222,232	121,705,753	92,176,746

[a] as calculated by summation of all the contig sizes

Table 3 - Contigs of ARES4, EGUFV2, GP and PTMG8 and GenBank accession numbers of closest matches, including *Puccinia psidii* (**in bold**)

Isolate and contig number	Contig length (Kb)	Genbank accession	Description	Identity (%)
ARES4_contig_10179	**795**	**KF431984**	***Puccinia* cf. *psidii* AE-2014 farnesyl-diphosphate farnesyltransferase gene**	**100**
ARES4_contig_38150	**609**	**KF432010**	***Puccinia* cf. *psidii* AE-2014 Sfi1 gene**	**91**
ARES4_contig_44461	908	FO906002	*Leptosphaeria maculans* lepidii ibcn84_scaffold00022	84
ARES4_contig_57517	**638**	**KF431984**	***Puccinia* cf. *psidii* AE-2014 farnesyl-diphosphate farnesyltransferase gene**	**90**
ARES4_contig_67468	**1190**	**KF432018**	***Puccinia* cf. *psidii* AE-2014 WD40 gene**	**83**
ARES4_contig_78583	596	EU090238	*Mycosphaerella graminicola* mitochondrion	86
ARES4_contig_78583	596	AY347307	*Penicillium marneffei* strain MP1 mitochondrion	89
ARES4_contig_78583	596	KF297618	*Beauveria pseudobassiana* strain C1010 mitochondrion	81
EGUFV2_contig_20848	1726	XM_002398648	*Moniliophthora perniciosa* FA553 hypothetical protein (MPER_00669) mRNA	92
EGUFV2_contig_29836	1530	XM_002396899	*Moniliophthora perniciosa* FA553 hypothetical protein (MPER_02719) mRNA	85
EGUFV2_contig_32062	**1489**	**KF431985**	***Puccinia* cf. *psidii* AE-2014 GIT_SHD gene**	**94**
EGUFV2_contig_32062	**1489**	**KF431996**	***Puccinia* cf. *psidii* AE-2014 pectate lyase 3 gene**	**96**
EGUFV2_contig_33682	724	HG970332	*Fusarium graminearum* chromosome 1	81
EGUFV2_contig_33682	724	XM_009256493	*Fusarium pseudograminearum* CS3096 hypothetical protein partial mRNA	81
EGUFV2_contig_33682	724	XM_381513	*Gibberella zeae* PH-1 hypothetical protein partial mRNA	81
EGUFV2_contig_40989	**652**	**KF432010**	***Puccinia* cf. *psidii* AE-2014 Sfi1 gene, complete cds**	**90**
EGUFV2_contig_43670	1852	XM_003303227	*Pyrenophora teres* f. teres 0-1 hypothetical protein, mRNA	96

EGUFV2_contig_59602	**1198**	**KF432004**	***Puccinia* cf. *psidii* AE-2014 ribosomal P0 like-protein gene,**	**85**
EGUFV2_contig_59602	**1198**	**KF431977**	***Puccinia* cf. *psidii* AE-2014 DNA repair protein rad1 gene**	**89**
EGUFV2_contig_83853	760	AM920464	*Penicillium chrysogenum* Wisconsin 54-1255 complete genome, contig Pc00c49	88
EGUFV2_contig_83942	627	XM_007678789	*Baudoinia compniacensis* UAMH 10762 hypothetical protein partial mRNA	93
EGUFV2_contig_83853	760	AM920464	*Penicillium chrysogenum* Wisconsin 54-1255 complete genome, contig Pc00c49	89
EGUFV2_contig_94117	562	JX910419	*Fusarium circinatum* mitochondrion	84
EGUFV2_contig_94117	562	KM486533	*Fusarium gerlachii* strain CBS 123666 culture-collection CBS:123666 mitochondrion	89
EGUFV2_contig_94117	562	HG970331	*Fusarium graminearum* mitochondrial	89
EGUFV2_contig_97243	**516**	**KF431974**	***Puccinia* cf. *psidii* AE-2014 ATP synthase complex subunit h gene**	**92**
EGUFV2_contig_97258	653	AB070824	*Aspergillus oryzae* sgtB gene for sugar transport protein	90
EGUFV2_contig_97258	653	XM_001822415	*Aspergillus oryzae* RIB40 hexose carrier protein, mRNA	90
EGUFV2_contig_97258	653	AP007162	*Aspergillus oryzae* RIB40 DNA, SC102	90
EGUFV2_contig_100371	794	AB525199	*Alternaria alternata* DNA, AMT genes region, strain: NBRC 8984, clone: AM-BAC-13	90
EGUFV2_contig_100371	794	AB525198	*Alternaria alternata* DNA, AMT genes region, strain: NBRC 8984, clone: AM-BAC-14	90
EGUFV2_contig_100716	783	XM_003854692	*Mycosphaerella graminicola* IPO323 putative Non-ribosomal peptide synthetase (NRPS1) mRNA	90
EGUFV2_contig_100716	783	XM_007933836	*Pseudocercospora fijiensis* CIRAD86 hypothetical protein partial mRNA	85
EGUFV2_contig_100958	699	XM_007692103	*Bipolaris oryzae* ATCC 44560 hypothetical protein partial mRNA	84
EGUFV2_contig_101384	663	XM_003855919	*Mycosphaerella graminicola* IPO323 isoleucine--tRNA ligase (MYCGRDRAFT_32217) mRNA	89
EGUFV2_contig_100187	676	XM_007918520	*Togninia minima* UCRPA7 putative gentisate -dioxygenase protein mRNA	84
EGUFV2_contig_100958	699	XM_007778815	*Coniosporium apollinis* CBS 100218 hypothetical protein partial mRNA	84

EGUFV2_contig_101321	640	XM_007682123	*Baudoinia compniacensis* UAMH 10762 hypothetical protein partial mRNA	97
EGUFV2_contig_101321	640	XM_007743715	*Cladophialophora psammophila* CBS 110553 hypothetical protein partial mRNA	94
GP_contig_18544	**768**	**KF432014**	***Puccinia* cf. *psidii* AE-2014 TFIIE beta-winged helix gene, complete cds**	**90**
GP_contig_62751	**509**	**KF432017**	***Puccinia* cf. *psidii* AE-2014 hypothetical protein gene, complete cds**	**89**
GP_contig_62937	**1263**	**KF432015**	***Puccinia* cf. *psidii* AE-2014 ubiquitin thiolesterase gene, complete cds**	**100**
GP_contig_74623	1504	FJ190628	*Cladosporium cladosporioides* isolate AFTOL-ID 1289 12S small subunit ribosomal RNA gene	97
GP_contig_74623	1504	GU250428	*Davidiellaceae* sp. CCFEE 5456 12S ribosomal RNA gene, partial sequence; mitochondrial	98
GP_contig_74623	1504	GU324020	*Dothideomycetes* sp. TRN 43 small subunit ribosomal RNA gene, partial sequence; mitochondrial	96
GP_contig_76526	834	KF169905	*Glarea lozoyensis* 74030 mitochondrion, complete genome	91
GP_contig_76526	834	KC832409	*Botryotinia fuckeliana* B05.10 mitochondrion, complete genome	89
GP_contig_76526	834	KF650574	*Rhynchosporium orthosporum* mitochondrion, complete genome	91
PTMG8_contig_28252	**1358**	**KF431996**	***Puccinia* cf. *psidii* AE-2014 pectate lyase 3 gene, partial cds**	**86**
PTMG8_contig_33596	**686**	**KF431979**	***Puccinia* cf. *psidii* AE-2014 clathrin associated protein AP47 gene**	**90**
PTMG8_contig_40496	**1028**	**KF431999**	***Puccinia* cf. *psidii* AE-2014 peptidase M17 gene**	**91**
PTMG8_contig_42746	**641**	**KF432002**	***Puccinia* cf. *psidii* AE-2014 PLN03108 gene**	**94**
PTMG8_contig_42746	**641**	**KF431979**	***Puccinia* cf. *psidii* AE-2014 clathrin associated protein AP47 gene**	**94**
PTMG8_contig_68127	**588**	**KF432014**	***Puccinia* cf. *psidii* AE-2014 TFIIE beta-winged helix gene**	**92**
PTMG8_contig_72063	**513**	**KF431984**	***Puccinia* cf. *psidii* AE-2014 farnesyl-diphosphate farnesyltransferase gene**	**100**
PTMG8_contig_93553	**774**	**KF431976**	***Puccinia* cf. *psidii* AE-2014 Cpn60/TCP1 gene**	**86**

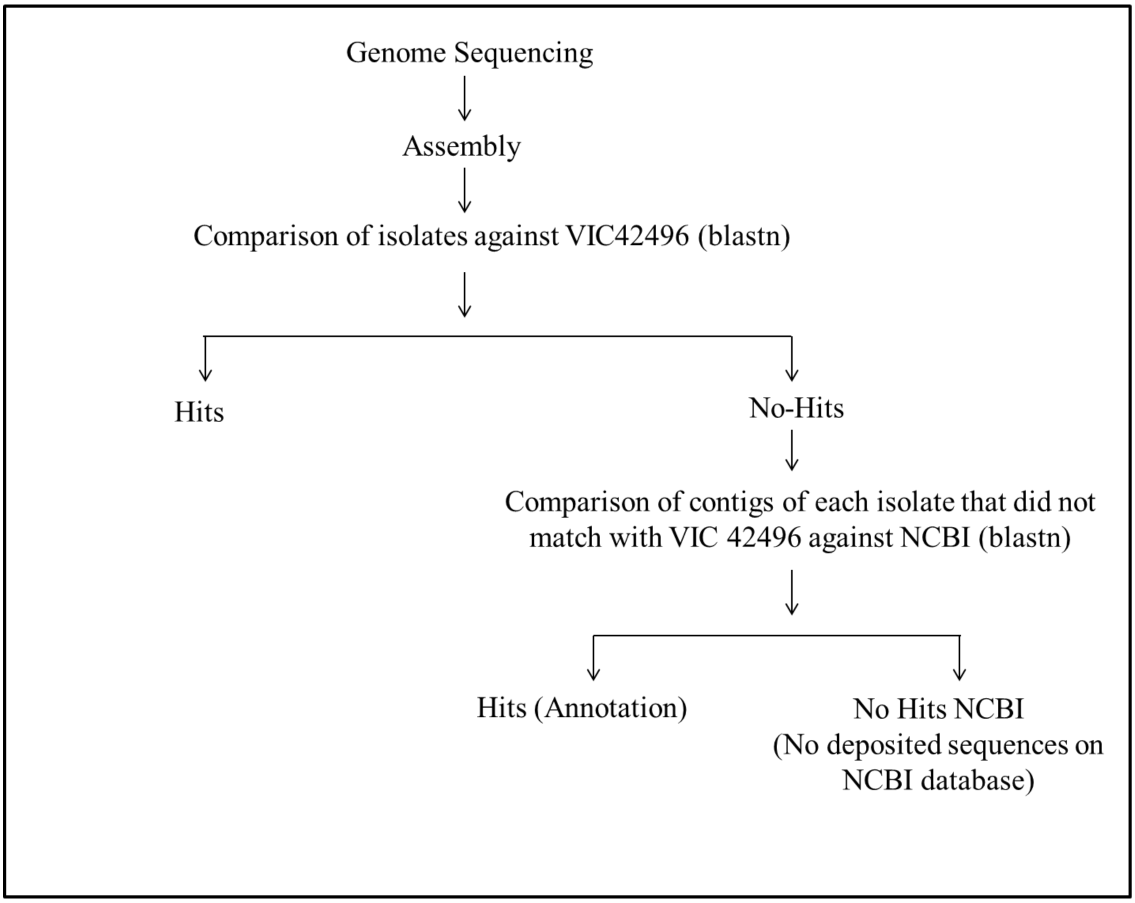

Figure 1 - Workflow of comparative genomic analysis of five isolates of *Puccinia psidii*

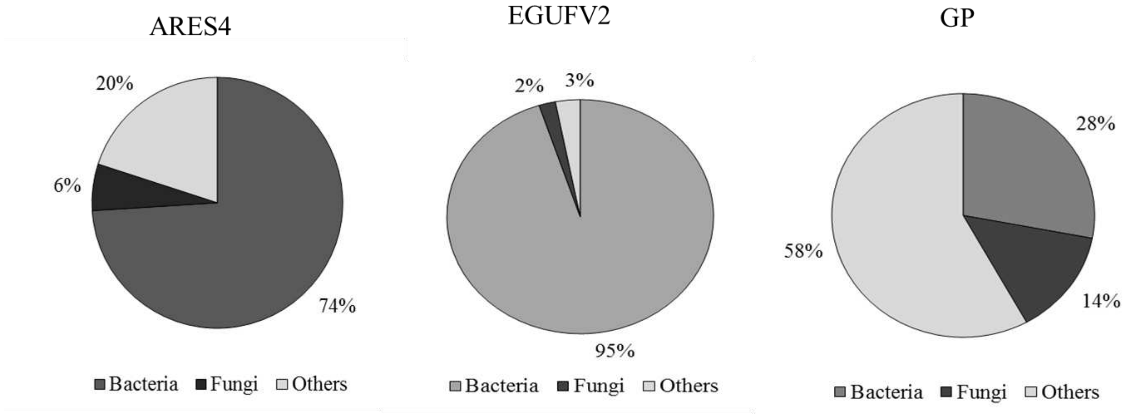

Figure 2 - Percentage of contigs of ARES4, EGUFV2 and GP that found matches to different organisms on NCBI database.