RODRIGO NEVES GRAÇA

GENETIC DIVERSITY OF *Puccinia psidii* POPULATIONS

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: 22 de fevereiro de 2011.

Prof. Eduardo Seiti Gomide Mizubuti
(Co-Orientador)

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Pesq. Ned Brian Klopfenstein

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(Orientador)

I dedicate this thesis to my parents, I love you.

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BIOGRAPHY

RODRIGO NEVES GRAÇA, son of Teodoro Alberto de Castro Graça and Laura Maria Neves Graça, has born on August 18 of 1979, in Belo Horizonte, Minas Gerais, Brazil. Bachelor in Agronomy (2004), Master (2007) and Doctorate (2011) in Plant Pathology by the University of Viçosa, Viçosa, Minas Gerais, Brazil.

SUMARY

RESUMO

GRAÇA, Rodrigo Neves, D. Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Diversidade genética de populações de *Puccinia psidii***. Orientador: Acelino Couto Alfenas. Co-orientadores: Eduardo Seiti Gomide Mizubuti e Olinto Liparini Pereira.

Relatou-se pela primeira vez a suplantação da resistência a ferrugem conferida pelo gene *Ppr-1* em *Eucalyptus grandis*, causada por uma nova raça de *Puccinia psidii* (raça 4), capaz de infectar uma ampla gama de clones resistentes à raça 1 predominate. Esta nova raça, e outras ainda não identificadas, devem ser consideradas na seleção de materiais resistentes à ferrugem. Até o presente, não havia estudos visando determinar a estrutura genética de populações de *P. psidii*. Assim, na segunda parte deste trabalho, baseado na análise de locos microsatélites constatou-se um indicativo de possível seleção de genótipos do patógeno em função da espécie hospedeira, além de evidências de reprodução asexuada em populações de *P. psidii* do Brasil. Estudos filogenéticas estão sendo conduzidos a fim de verificar a presença de espécies crípiticas associadas a espécies hospedeiras específicas. Na terceira parte deste trabalho, isolados coletados em diferentes regiões do Brasil, tiveram o perfil oito locos microsatelites comparados com isolados do Paraguai, Uruguai e EUA (Califórnia e Havaí). Genótipos multilocus (GM) de *P. psidii* coletados em diferentes espécies hospedeiras na América do Sul indicaram que a espécie hospedeira afeta significativamente a estrutura genética de *P. psidii*, como relatado no artigo dois. Ao contrário dos isolados da América do Sul, todos os 50 isolados coletados em nove hospedeiros em quatro ilhas do Havaí, compartilharam um único GM, indicando a recente introdução de um único genótipo do fungo

em quatro ilhas do Havaí, como previamente hipotetizado. A presença de um único GM em diferentes espécies hospedeiras indica ausência de seleção por hospedeiro na população de ferrugem do Havaí. O mesmo GM detectado em todos os isolados do Havaí foi detectado também em duas espécies hospedeiras na Califórnia, indicando uma origem comun do inóculo de *P. psidii*. Entretanto, a origem do GM invasor ainda não foi determinada.

ABSTRACT

GRAÇA, Rodrigo Neves, D. Sc., Universidade Federal de Viçosa, February, 2011. **Genetic diversity of *Puccinia psidii* populations**. Adviser: Acelino Couto Alfenas. Co-advisers: Eduardo Seiti Gomide Mizubuti and Olinto Liparini Pereira.

We reported for the first time the breakdown of the rust resistance gene *Ppr-1* in *Eucalyptus grandis*, caused by a new race of *Puccinia psidii* (race 4), capable to infect a large number of clones resistant to race 1 predominant. This new race and other unrecognized pathogen races should be strongly considered when selecting clones resistant to the rust. So far, no studies have been directed toward determining *P. psidii* population genetic structure, which is essential to understand the physiologic variability and the pathogen evolution mechanisms. So, in the second part of this work, based on the analysis of microsatellite loci, we detected evidences of strong selection of pathogen genotypes by host species, and also indicatives of high rate of asexual reproduction on *P. psidii* population from Brazil. Phylogenetic studies are underway to test the hypothesis of presence of cryptic species associated within each host. In the third part of this study, rust isolates collected on different regions in Brazil, were compared based on eight microsatellite loci to isolates from Paraguay, Uruguay, and USA (California and Hawaii). Multilocus genotypes (MG) of *P. psidii* isolates collected on different host species in South America indicated that host species strongly influence the rust population structure, as observed on chapter two. In contrast with the South American groups, all 50 rust isolates collected on nine different hosts in four Hawaiian Islands, share a single unique MG, indicating a recent introduction of a single rust genotype on four Hawaiian Islands, as previously hypothesized. The presence of a single MG across different host species indicates lack of selection by host on the rust population in Hawaii. The same unique MG detected across all Hawaiian isolates was also detected on two host species in California, indicating a common origim of the *P. psidii* inoculum to both locations; however, the geographic origin of the invasive MG has not yet been determined.

GENERAL INTRODUCTION

The Myrtaceous rust caused by *Puccinia psidii* Winter was originally reported in 1884 on *Psidium guajava* (= *Psidium pomiferum*) in Southern Brazil (Maclachlan, 1938), and has been found infecting approximately 70 Myratceae species mainly in tropical and subtropical areas (Farr and Rossman, 2010). The main signals of this disease are characterized by yellow uredinial pustules produced on young-leaves, shoots, stems, floral buds, and young fruits (Coutinho et al., 1998; Tommerup et al., 2003; Alfenas et al., 2009). The life cycle of *P. psidii* is not completely understood, but it is considered an autoecious and macrocyclic rust (Tommerup et al., 2003; Glen et al., 2007). All spore stages are formed on the same host, except the spermogonial stage, which has never been observed and is apparently not produced. Infections by haploid basidiospores were observed in *S. jambos*; however, it remains unknown how dikaryotization occurs (Figueiredo, 2001). Conclusive evidence of sexual reproduction in *P. psidii* populations is essential for inferences about its evolution. Pathogens that reproduce asexually depend mostly on mutation (or other asexual recombination processes) for creating new genotypes, thus disease control through resistance tends to be more effective (McDonald and Linde, 2002; Milgroom and Fry, 1997).

This rust fungus is considered one of the most important eucalypt pathogens in South America (Coutinho et al., 1998; Rayachhetry et al., 2001; Tommerup et al., 2003; Glen et al., 2007). On *Eucalyptus* spp., necrosis and hypertrophy on twigs and branch tips can occur, causing growth loss and branch multiplication (Coutinho et al., 1998; Booth et al., 2000; Alfenas et al., 2009). Wood volume losses of up to 41% have been attributed to *P. psidii* infection (Takahashi, 2002). Although this rust rarely kills its host, mortality has resulted from infection of highly susceptible *Eucalyptus* spp. and *Syzygium jambos* genotypes (Coutinho et al., 1998; Glen et al., 2007; Uchida et al., 2006). In *E. grandis*, resistance is controlled by a

locus of main effect, which makes this species useful in breeding programs (Junghans et al., 2003). However, based on the pattern of inheritance, it is possible that changes in the pathogen's genetic structure can make the host-resistance gene ineffective.

Puccinia psidii is expanding its geographic distribution, and based on the potential damage to diverse myrtaceous species, it is considered a major potential threat to the Myrtaceae in Southeast Asia and surrounding islands, Australia, and South Africa (Ciesla et al., 1996; Coutinho et al., 1998; Tommerup et al., 2003; Glen et al., 2007; Carnegie et al., 2010). In Hawaii, *P. psidii* infects several native and introduced species of Myrtaceae including, an endemic tree species known as 'ohi'a, the dominant tree species in Hawaii's remnant native forests (Uchida et al., 2006; Loope, 2010). Of present concern is the potential introduction of new pathogen genotypes into Hawaiian Islands. More aggressive isolates may cause even more damage to the native forest of Hawaii. Despite *P. psidii* importance in South America and Hawaii, and its potential damage to Myrtaceae species worldwide, there is no information about the genetic structure of *P. psidii* global populations. This information is essential for inferences about pathogen migratory routes and sources of introduction.

This thesis comprisses three articles, aiming to understand the genetic basis of *P. psidii* populations. In article 1, two hypotheses were tested to explain the incidence of rust on the eucalypt clone BA6021, previously classified as resistant to the isolate UFV2 (race-1; Xavier, 2002): 1) clonal admixture, plants infected with rust were not truly representative of clone BA6021; and 2) genetic variability on *P. psidii* populations, a new pathogen race had emerged with the capability to overcome the resistance mechanisms of the clone BA6021. In addition, we tested if the potentially new *P. psidii* race was capable of overcoming resistance conferred by the *Ppr-1* gene in *E. grandis*. In the second article, we used microsatellite markers to infer about the genetic structure of *P. psidii* populations from different host species in Brazil, which is believed to be the putative diversity center for this rust (Tommerup et al., 2003; Loope, 2010). We also used linkage disequilibrium tests to infer about the possible occurrence of sexual reproduction among Brazilian rust isolates. In article three, we analyzed microsatellite loci to determine the genetic relationship among rust isolates from South America, California and Hawaii. We examine the hypotheses that *P. psidii* was introduced into Hawaii by trade of rust infected myrtaceous plants from California, and that *P. psidii* populations from South America are differentiated from the rust populations found in California and Hawaii, as result of geographic isolation.

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1. ARTICLE ONE

A new race of *Puccinia psidii* defeats the *Ppr-1* resistance gene in *Eucalyptus grandis*

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ABSTRACT

Rust caused by *Puccinia psidii* is one the most destructive diseases of *Eucalyptus*. Management of the disease is achieved through selection of resistant host genotypes. Recently, eucalypt plants from clone BA6021, resistant to *P. psidii* isolate race-1, were infected by rust in Brazil. Microsatellite profiles of infected plants confirmed that the host was indeed clone BA6021. In pathogenicity tests, the resistant clones BA6021 and G21 (which carry the resistance gene *Ppr-1*) were found susceptible to the newly discovered isolate EUBA-1, indicating a new biotype of the pathogen. These results show that the isolate EUBA-1 and other potentially unrecognized pathogen races should be given strong consideration for eucalypt breeding programs aimed rust resistance.

Keywords: *Eucalyptus*, rust, resistance gene, variability, race

Introduction

Rust caused by *Puccinia psidii* Winter is a limiting factor to eucalypt production in Brazil, where the disease has caused up to 41% wood-volume losses in some plantations (Takahashi 2002). This disease can cause growth reduction, apical death, and even kill young plants intended for planting (Alfenas *et al.* 2009). Besides eucalypt, this pathogen also infects at least 70 other myrtaceous species (Farr and Rossman 2010). Because this rust pathogen poses a high potential for damage to the Myrtaceae family and its capacity to be dispersed over long distances, *P. psidii* is considered a serious threat to the native flora of Australia and surrounding islands and to commercial eucalypt plantations in South Africa (Glen *et al.* 2007).

The high inter- and intra-specific genetic variability for rust resistance in *Eucalyptus* species has allowed disease control through selection and planting of cuttings, seedlings, or species with resistance to *P. psidii* (Alfenas *et al.* 1997, Carvalho *et al.* 1998, Dianese *et al.* 1984, Tommerup *et al.* 2003). In a particular *E. grandis* family, the rust resistance is controlled by a single major gene, *Ppr-1* (*Puccinia psidii* resistance gene 1), with variable expression depending on the host genetic background (Junghans *et al* 2003a). According to this pattern of inheritance, slight changes in the genetic structure of the pathogen could be enough to overcome this resistance gene.

Because urediniospores are easily dispersed over long distances and have the capacity to form large effective populations, as observed with other rusts, *P. psidii* is expected to have a high genetic variability and consequently a high evolutionary potential. Several cross-inoculation studies using Myrtaceae species have indicated the physiologic differences among *P. psidii* populations (Maclachlan 1938, Joffily 1944, Ferreira 1981, Castro *et al.* 1983, Coutinho and Figueiredo 1984, Coelho *et al.* 2001, Aparecido *et al.* 2003), . Considering that *P. psidii* is a biotrophic pathogen that is capable of infecting different Myrtaceae species across a wide geographic range, it appears reasonable that host-specific virulence genes could have been selected within the pathogen populations.

In April 2008, yellow uredinial pustules of *P. psidii* were observed on clone BA6021 (*E. grandis* hybrid of Rio Claro, SP) plants in Southern Bahia, Brazil. In previous studies (data not shown), this clone had been previously classified as resistant to the isolate UFV-2 (race 1), which was commonly used to select rust-resistant eucalypt genotypes in Brazil (Junghans *et al.* 2003a, Xavier 2002). Two hypotheses were tested to explain the incidence of rust on the clone BA6021: 1) clonal admixture, plants infected with rust in South Bahia were

not truly representative of clone BA6021; and 2) genetic variability within/among *P. psidii* populations, exemplified by the emergence of a new pathogen race with the capability to overcome the resistance mechanisms of the clone BA6021. An additional objective of the present study was to verify if the potentially new *P. psidii* race was capable of overcoming resistance conferred by the *Ppr-1* gene in *E. grandis*.

Material and methods

Clonal admixture

To evaluate the hypothesis of clonal admixture, cuttings from the clone BA6021 were collected from four separate forestry nurseries located in different Brazilian states. DNA was extracted from each cutting, and cuttings were genotyped using 15 microsatellite (SSR) markers (Brondani *et al.* 1998). PCR amplifications were performed in 15-µL reaction volumes with an initial denaturation step at 94ºC for 5 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 58ºC for primer annealing and 1 min at 72ºC for DNA extension; and a final extension at 72ºC for 15 min. Final reagent concentrations were 30 ng template DNA, 2.5 mM dNTPs, 0.25 units of Taq DNA polymerase (Applied Biosystems), 10 µM of each primer, 1.5 µL of 10x PCR buffer and 6.15 µL of MiliQ water. The amplified DNA fragments were separated on a 10% (w/v) polyacrylamide gel (29:1 acrylamide/bis-acrylamide). A 10-bp DNA ladder (Life Technologies) was used to score the allele sizes.

Puccinia psidii isolates

Two single-uredinial isolates were used in this study: isolate UFV-2, collected from young *E. grandis* plantations in São Paulo, Brazil (Junghans *et al.* 2003a, Xavier 2002), and the isolate EUBA-1, collected on cuttings of clone BA6021 (hybrid *E. grandis* x *E. urophylla*) from a forest nursery in southern Bahia, Brazil. Single-pustule-derived urediniospores of each isolate were multiplied on the young leaves of *Syzygium jambos* cuttings (Ruiz *et al.* 1989). After 12 days, the newly produced urediniospores were collected and stored in 1.5-mL Eppendorf® tubes at -80ºC. The isolates were used for tests within 30 days of multiplication on *S. jambos*. To assure isolate purity, isolates were inoculated on eucalypt clones on different days. The inoculated plants were physically separated under controlled conditions until the end of the experiment (Ruiz *et al.* 1989).

Plant material

Seven eucalypt clones (G-21, G-26, BA-6021, 1183, 847, 3918, and 1205) from different species were inoculated with *P. psidii* (Table 1). Of these, *E. grandis* clones G21, G26, and G38 were used in a previous study of rust inheritance (Junghans *et al.* 2003a), and clone BA6021 was previously classified as resistant to the UFV-2 isolate (data not shown), but recently found to be infected with *P. psidii* in southern Bahia. Ten cuttings of each clone were transplanted to 2-L pots containing the substrate MecPlant® supplemented with 6 $Kg.m^{-3}$ of simple super phosphate and 3 $Kg.m^{-3}$ of Osmocote® (19N-6P-10K). The plants were inoculated after 30-days growth in the pots.

Physiologic difference between *Puccinia psidii* isolates

Cuttings from the *E. grandis* clone BA6021 (clonal identity was confirmed by microsatellite markers, results not shown) were inoculated with the isolate UFV-2 or EUBA-1. Both isolates were also used to inoculate two clones (*E. urophylla* clone 1183 and *E. grandis* clone 3918) that were previously classified as susceptible to the isolate UFV-2, and four clones (*E. grandis* clone G21, *E. grandis* clone G26, *E. grandis* clone 1205, and *E. urophylla* clone 847) that were previously classified as resistant to the isolate UFV-2. The clone G21 is heterozygous for the rust-resistance gene *Ppr-1* (Junghans *et al.* 2003a). The eucalypt plants were inoculated with a suspension of $2x10^4$ urediniospores mL^{-1} (Ruiz *et al.* 1989). Ten plants of each clone were inoculated with the isolate UFV-2, and ten plants of each clone were inoculated with the isolate EUBA-1. To avoid cross-contamination of isolates, the inoculations were conducted on different days, and the plants were kept physically separated in mist and growth chambers. The inoculum suspension of each isolate was uniformly sprayed on both surfaces of young leaves, using a nº 15 De Vilbss, with an electrical compressor at 0.8 kgf cm^{-2}. After inoculation, plants were kept for 24 h in a mist 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 (Ruiz *et al.* 1989). To assure uniform inoculation and validate inoculum viability, five *S. jambos* cuttings were utilized as susceptible controls for each isolate.

At 20 days post-inoculation, rust severity was evaluated using a disease rating system with four severity scores (Junghans *et al.* 2003b): S0 = immunity or hypersensitive reaction (HR); S1 = punctiform pustules, < 0.8 mm; S2 = medium pustules, from 0.8 to 1.6 mm; and S3 = large pustules, > 1.6 mm, and in some cases with pustules on the leaf petioles and young

branch. Plants classified as S0 or S1 were considered resistant, and plants classified as S2 or S3 were considered susceptible. Two independent experiments were conducted at different times.

Puccinia psidii isolates aggressiveness on eucalypt

The aggressiveness of *Puccinia psidii* isolates, UFV-2 and EUBA-1, was evaluated through inoculation on the same seven clones used in the physiologic difference study (Table 1). The same experimental design was used, including the same number of replications. The aggressiveness of both isolates was assessed within 12 and 20 days post-inoculation. Disease severity was evaluated on the second leaf pair, counted from the plant apices to the base, using the disease rating system of Junghans *et al.* (2003b). Digital pictures were analyzed using the software Quant® to determine the percentage of leaf area with lesions (Vale *et al.* 2003). To count the number of urediniospores produced in 1 cm^2 of leaf area (Ruiz *et al.* 1989), three circular segments (1.2 cm) removed from the central part of the first or second leaf were placed in glass vials with 3 mL distilled water plus 2% Tween 20. The vials containing the leaf segments were mixed in a vortex for 1.5 min and the number of urediniospores was determined using a Neubauer chamber (hemacytometer). Two evaluations were made for each vial. Data analyses were performed with the software Statistica ® 7.0. Two independent experiments were conducted at different times.

Results and discussion

Clonal admixture

The hypothesis of clonal mixture was rejected based on the multilocus genetic profiles in polyacrylamide gel of the four sources of the clone BA6021, which showed an identical genetic profile for all. It was concluded that the eucalypt genotype infected by *P. psidii* in southern Bahia was indeed the clone BA6021, which was previously classified as resistant to the isolate UFV-2 (race 1).

Physiologic difference between *Puccinia psidii* isolates

Of seven clones inoculated with *P. psidii,* three (847, 1205, and G26) were classified as resistant, and two (1183 and 3918) were classified as susceptible to both isolates, UFV-2 and EUBA-1 (Table 1). However, two clones (BA6021 and G21) were found to be resistant to isolate UFV-2, but susceptible to isolate EUBA-1 (Table 1). This result further confirms the existence of physiologic difference within *P. psidii* populations (Aparecido *et al.* 2003, Castro *et al.* 1983, Coelho *et al.* 2001, Coutinho and Figueiredo 1984, Ferreira 1981, Joffily 1944, Maclachlan 1938). Three *P. psidii* races have been previously reported in Brazil (Xavier 2002); however, the isolate EUBA-1 represents a fourth pathogen race in Brazil, based on our results. Clones resistant to the isolate EUBA-1 and susceptible to UFV-2 were not observed. Therefore, isolate EUBA-1 was virulent on a greater number of eucalypt genotypes, of the seven clones tested here.

Based on previously studies, the genotypes G21 and G26 were previously classified as resistant to 21 *P. psidii* isolates collected from different locations in Brazil, which included the isolate UFV-2. In those studies, the G21 and G26 clones were rated as S0, showing hypersensitive reactions, and it was further demonstrated that the rust resistance on clone G21 is controlled by a single dominant gene, *Ppr-1*, *Puccinia psidii* resistance gene-1, which is heterozygous in clone G21 (Junghans *et al.* 2003a). In the current study, clone G21 was still resistant to the isolate UFV-2, and exhibited a hypersensitive response. However, clone G21 was susceptible to the isolate EUBA-1, with a rust rating of S2. This is the first report of the break-down of rust resistance linked to gene *Ppr-1*in eucalypts. The breakdown of resistance in clone G21 has been demonstrated only through artificial inoculations, and a similar breakdown of resistance in clone G21 in the field has not been reported to date. It should be noted that eucalypt clone G21 is only planted in Sao Paulo state, and the new rust race (represented by EUBA-1) was found in southern Bahia, where the the clone G21 is not

planted in plantations. Based on this information, it appears that urediniospores of *P. psidii* EUBA-1 isolate have not yet migrated from South Bahia to Sao Paulo.

Within the clones resistant to both isolates, clone G26, previously classified as resistant to the isolate UFV-2 (Junghans *et al.* 2003a), showed small pustules (S1 score) surrounded by a chlorotic halo after inoculation with the EUBA-1 isolate. Therefore, the EUBA-1 isolate showed a capacity to partially overcome the initial defense mechanisms of clone G26. Clones 847 and 1205 were classified as resistant (S0 score) when inoculated with both isolates; clone 847 showed a strong hypersensitive response, and clone 1205 exhibited tiny chlorotic spots or "flecks".

Puccinia psidii isolates aggressiveness on eucalypt

Analysis of variance showed significant effects ($P \leq 0.01$) of isolates and isolate x clone on the percentage of leaf area with lesions at 12 days post-inoculation (Table 2), indicating that different rust isolates produced different responses when inoculated on the same eucalypt clone. No significant differences in disease severity were observed among clones BA6021, 1183, and 3918 when inoculated with the EUBA-1 isolate; these three clones were all susceptible to the EUBA-1 isolate (Figure 1). However, only the clones 1183 and 3918 were susceptible to inoculations with the UFV-2 isolate, showing 6.7 and 7.8% leaf area with lesions, respectively. Clone BA6021 was classified as resistant to the UFV-2 isolate (Figure 1). Significant effects ($P \leq 0.01$) on leaf area with lesions were observed at 12 days post-inoculation for *P. psidii* isolates and eucalypt clones. However, no significant effects on clone x isolate were observed at 20 days post-inoculation (Table 2).

Puccinia psidii urediniospores were observed on the clones G21, G26, 6021, 3918, and 1183, at 12 and 20 days post-inoculation with the EUBA-1 isolate. However, when inoculated with the UFV-2 isolate, *P. psidii* urediniospores were observed only on the clones 3918 and 1183, at 12 and 20 days post-inoculation (Figure 2). At 12 days post-inoculation, a larger number of urediniospores ($6.7x10^4$ urediniospores.cm^{-2} of leaf area) were produced on the clone 3918 when inoculated with the EUBA-1 isolate, compared to urediniospores ($1.25x10^4$ urediniospores.cm^{-2} of leaf area) produced by the UFV-2 isolate (Figure 2). Thus, the EUBA-1 isolate appears to be more aggressive than the UFV-2 isolate on clone 3918. The fact that the EUBA-1 isolate was more aggressive on the eucalypt clones tested and virulent on a larger number of clones, indicates that the EUBA-1 isolate, and possibly other undiscovered races, are important factors for consideration in eucalypt breeding programs to increase rust

resistance. However, despite the observed difference in virulence and aggressiveness between the isolates EUBA-1 and UFV-2, based on ten *P. psidii* microsatellite markers (Zhong et al. 2008) this two isolates had identical multilocus genotypes (unpublished data). The discovery of a new pathogen race, more aggressive and virulent to larger number of eucalypt clones, reinforces the need to include as broad a range as possible of *P. psidii* isolates in the selection of resistant eucalypt genotypes. Furthermore, population genetic studies aimed at understanding the distribution of genetic variability within pathogen populations from different hosts and geographic locations are essential for any programs directed toward managing or better understanding eucalypt rust disease.

Conclusions

The *P. psidii* isolate EUBA-1 was able to overcome the rust-resistance gene *Ppr-1* in *E. grandis* (clone G21), therefore this isolate belongs to a new pathogen race, named here as race 4.

The EUBA-1 isolate (race 4) was also able to infect plants from the clone BA6021, which was previously classified as resistant to the isolate UFV-2 (race 1).

The isolates EUBA-1 (race 4) and UFV-2 (race 1) differ in virulence, and EUBA-1 displays virulence on a larger number of eucalypt clones.

The isolate EUBA-1 (race 4) is more aggressive than the isolate UFV-2 on the clone 3918.

Acknowledgments

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Table 1. *Eucalyptus* spp. clones rated based on rust resistance, according to the disease rating system proposed by Junghans et al. (2003b), 12 and 20 days after inoculation with the *Puccinia psidii* isolates UFV-2 and EUBA-1.

Clones	12 Days		20 Days	
	UFV-2	EUBA-1	UFV-2	EUBA-1
G-21	HR	S1	HR	S2
G-26	HR	S1	HR	S1
BA-6021	HR	S3	HR	S3
1183	S3	S3	S3	S3
847	HR	HR	HR	HR
3918	S2	S3	S3	S3
1205	HR	HR	HR	HR

* HR, S0 and S1 = resistant; and S2 and S3 = Susceptible

Table 2. Analysis of variance on percentage area with lesions, 12 and 20 days post-inoculation with the *Puccinia psidii* isolates UFV-2 and EUBA-1 on different eucalypt clones.

Source	**df**	***F* value**	***P***
12 days post-inoculation			
Isolate	1	32.95	0.0001
Clone	6	0.85	0.3689
Isolate x Clone	6	9.73	0.0066
20 days post-inoculation			
Isolate	1	31.48	0.0001
Clone	6	16.69	0.0001
Isolate x Clone	6	0.31	0.5869

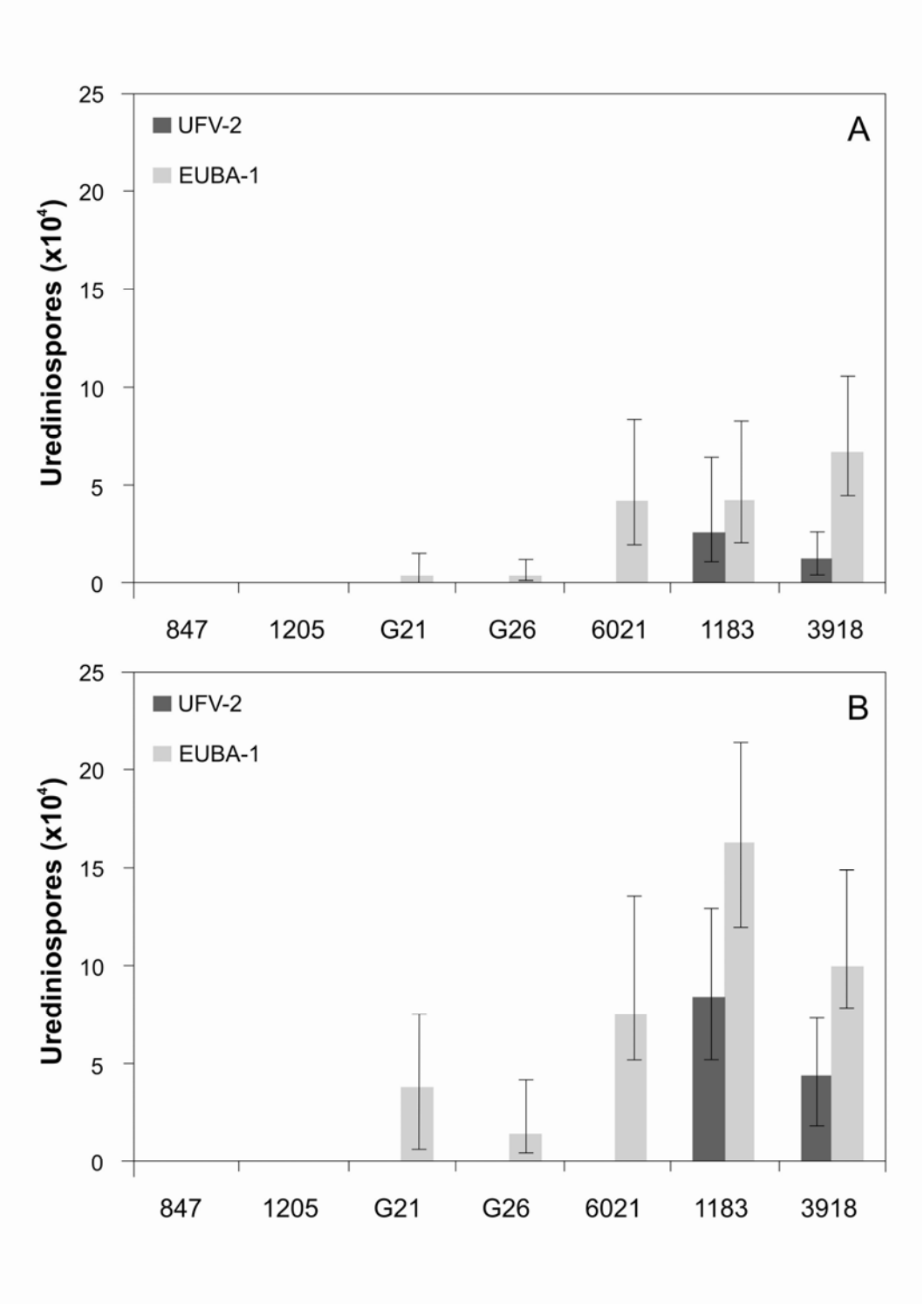

Figure 1. Leaf area with rust 12 (A) and 20 days (B) after inoculation with *Puccinia psidii*. Error bars correspond to ± confidence interval.

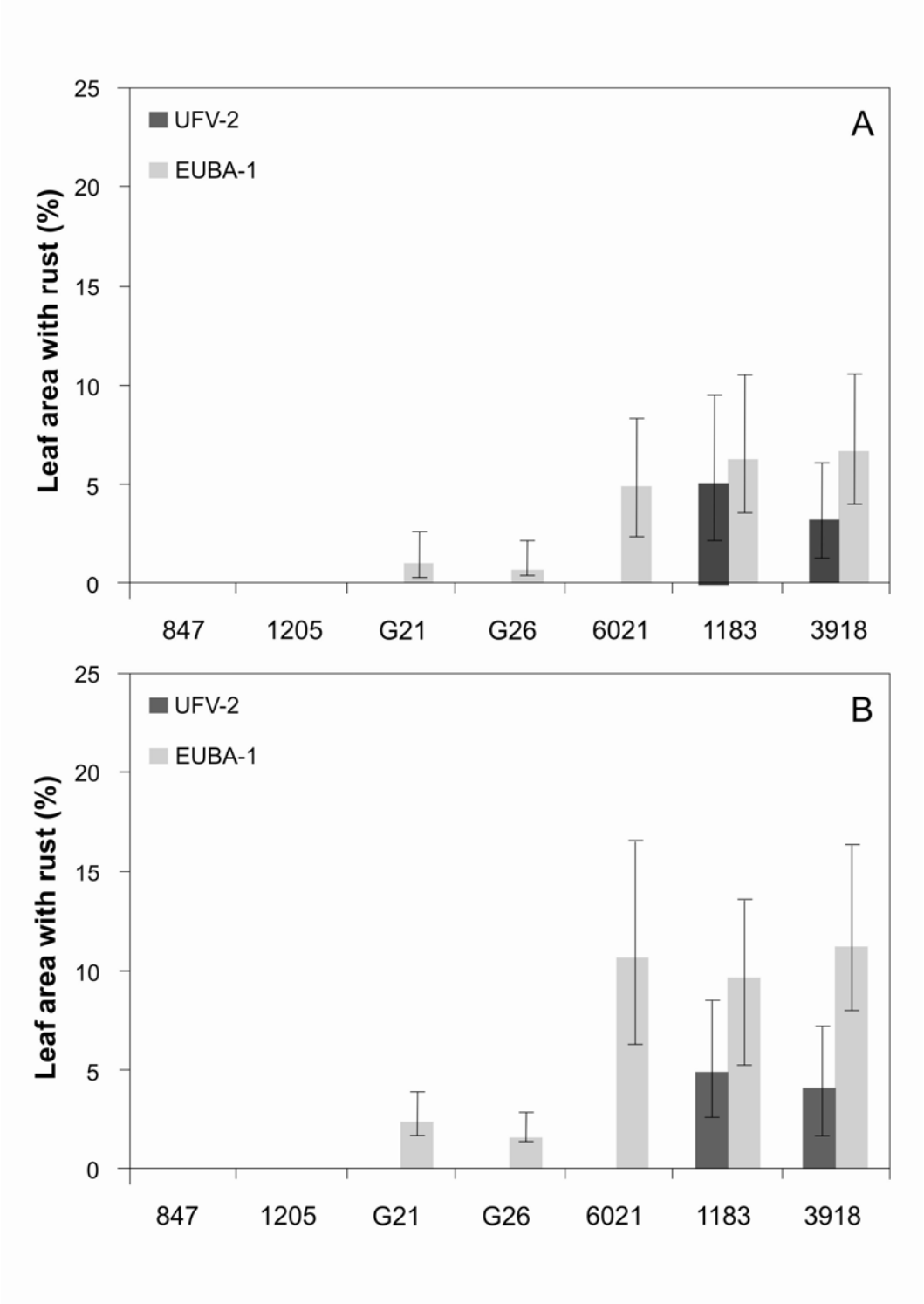

Figure 2. Number of urediniospores (x10^4) produced for 1 cm^2 of leaf area, 12 (A) and 20 (B) days after inoculation with *Puccinia psidii*. Error bars correspond to ± confidence interval.

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2. ARTICLE TWO

Multilocus genotypes indicate selection by host in *Puccinia psidii* populations from Brazil

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Abstract

Population genetic approaches were used to determine the genetic structure of the rust fungus *Puccinia psidii* in Brazil, believed to be the putative center of pathogen diversity. Ten microsatellite markers were used to examine the amount and distribution of genetic variability of 148 rust isolates collected on seven Myrtaceae hosts across a wide geographic area. Analysis of molecular variance indicated no genetic differentiation among isolates from different geographic locations, and high differentiation between isolates from different hosts (97%, $P > 0.001$). Principal coordinate plots, also indicated high degree of genetic differentiation among isolates collected on different host species, revealing five major groups. The Neighbor Joining tree also clustered the rust isolates on five groups, based on host of origin. The high proportion of repeated multilocus genotypes within each host, combined with

high values of I_A and r_D, and low values of F_{IS}, indicated clonal population structure. The haplotype MJ-Network also supported the hypothesis of host selection and clonal reproduction. Microsatellite data indicate occurrence of selection by host and high rate of clonal reproduction of *P. psidii* population from Brazil.

Introduction

Puccinia psidii (Winter) is a biotrophic pathogen (Basidiomycetes, Uredinales), commonly referred to as guava or eucalypt rust, that infects nearly 20 plant genera and 70 species, primarily in the Myrtaceae (Farr and Rossman, 2010). The disease main signals are yellow uredinial pustules produced on young-leaf, shoot, stem, floral bud, and fruit (Coutinho et al., 1998; Tommerup et al., 2003; Alfenas et al., 2009). On *Eucalyptus* spp., necrosis and hypertrophy on twigs and branch tips can occur, causing growth loss and branch multiplication (Coutinho et al., 1998; Booth et al., 2000; Alfenas et al., 2009). Wood volume losses of up to 41% have been attributed to *P. psidii* infection (Takahashi, 2002). Although this rust rarely kills its host, mortality has resulted from infection of highly susceptible *Eucalyptus* spp. and *Syzygium jambos* genotypes (Coutinho et al., 1998; Glen et al., 2007; Uchida et al., 2006; Loope, 2010). This rust is considered one of the most important eucalypt pathogens in South America (Coutinho et al., 1998; Rayachhetry et al., 2001; Tommerup et al., 2003; Glen et al., 2007). Breeding programs are underway to improve resistance of eucalypts to *P. psidii*. In *E. grandis*, resistance is controlled by a locus of main effect, which makes this species useful in breeding programs (Junghans et al., 2003). However, based on the pattern of inheritance, slight changes in the pathogen's genetic structure can overcome this host resistance gene and render it ineffective (Graça et al, 2011).

Urediniospores are the primary means of pathogen spread. Dispersion of rusts in general is mediated primarily by wind-dispersed urediniospores, but long distance spread is also associated with movement of infected plant material (Isard et al., 2005; Brown and Hovmøller, 2002). Rust pathogens generally produce urediniospores capable of spreading long distances (Isard et al., 2005; Brown and Hovmøller, 2002) and presumably forming large effective population sizes. With such capacity, *P. psidii* is perhaps able to colonize and adapt to new hosts and environments. Cross inoculation studies with several myrtaceous species demonstrated physiological variability among *P. psidii* populations (Aparecido et al., 2003; Castro et al., 1983; Coelho et al., 2001; Coutinho and Figueiredo, 1984; Ferreira, 1981; Maclachlan, 1938; Joffily, 1944; Xavier, 2002). Because *P. psidii* is widely distributed and

infects several species of Myrtaceae, it seems reasonable that specific host species could be selecting for specific virulence genes in the pathogen populations (Xavier, 2002). However, despite the reports of physiological variability within *P. psidii*, only limited molecular based information is available to assess genetic variability in rust populations (Zhong et al., 2008). Furthermore, little is known about the mechanisms driving genetic variability, such as mutation, gene flow, selection, genetic drift, and sexual recombination. Baseline information on the pathogen's genetic structure is essential to support the selection of resistant host genotypes and decrease the risk of host resistance breakdown (Peever et al., 2000).

The life cycle of *P. psidii* is not completely understood. It is considered to be an autoecious and macrocyclic rust species (Tommerup et al., 2003; Glen et al., 2007). All spore stages are formed on the same host, except the spermogonial stage which has never been observed and is apparently not produced. Infections by haploid basidiospores were observed in *S. jambos*, however, it remains unknown how dikaryotization occurs (Figueiredo, 2001). Conclusive evidence of sexual reproduction in *P. psidii* populations is essential for inferences about its evolution. Pathogens that reproduce by asexual means depend mostly on mutation (or other asexual recombination process) for creating new genotypes, thus disease control through resistance tends to be more effective (McDonald and Linde, 2002; Milgroom and Fry, 1997). Molecular markers provide the best tools for inferring the rate of clonal reproduction in pathogen populations (Taylor et al., 1999; Halkett et al., 2005).

Despite the potential threats of *P. psidii* to many forest ecosystems world-wide and its expanding geographic range, little is known about the genetic structure of populations of this pathogen in Brazil, which is believed to be the putative diversity center of the rust (Tommerup et al., 2003). The main objectives of this study were (1) to determine the genetic relationships among rust isolates from different host species and geographic locations in Brazil, (2) determine the influence of host selection on *P. psidii* genotypes, and (3) provide inferences about the extent and evolutionary effects of sexual and asexual reproduction within these populations.

Material and Methods

Sampling

Single uredinial isolates of *P. psidii* were collected across nine Brazilian states on the following Myrtaceae species: *Eucalyptus* spp. (EU), *Psidium guajava* (PG), *P. araca* (PA), *Syzigium jambos* (SJ), *S. cumini* (SC), *Myrciaria cauliflora* (MC), and *Eugenia uniflora* (EG)

(Figure 1; Table 1). An additional isolate was collected from EU in Uruguay. A total of 146 isolates was collected from March of 2008 to August of 2009. Because *P. psidii* infects only young tissues, the number of isolates collected on each state and host varied due to differences of microclimatic conditions, host prevalence, and the phenological state of host organs. All survey points were georeferenced using a GPS (Figure 1; Table S1). Each isolate was composed by three distinct single uredinial pustules (> 0.6 mm of diameter) collected on the same plant on each location. The single pustules were placed separately in 2-mL Eppendorf® vials, which were stored at -80°C prior to DNA extraction. One pustule from each isolate was used for DNA extractions, and the remaining two were kept at -80°C. Besides the 146 isolates collected, we genotyped two other isolates, the isolate UFV-2 race-1 (Xavier et al., 2002) and the isolate EUBA-1, the latter one demonstrated the capacity to defeat the resistance gene *Ppr-1* on *E. grandis* (Graça et al., 2011). A total of 148 isolates was analyzed for this study.

Microsatellite genotyping

We genotyped 148 isolates at 10 microsatellite loci: *PpSSR*012, *PpSSR*014, *PpSSR*018, *PpSSR*022, *PpSSR*087, *PpSSR*102, *PpSSR*146, *PpSSR*161, *PpSSR*178, and *PpSSR*195; originally developed from a genomic DNA library enriched for a SSR motif of AG (Zhong et al., 2008; Table 2). To amplify the following loci: *PpSSR*022, *PpSSR*087, *PpSSR*102, *PpSSR*146, *PpSSR*178, and *PpSSR*195, we designed new primers (Table 2), based on the *P. psidii* microsatellite sequences in the GenBank (Zhong et al., 2008) using the Primer3 (Rozen and Skaletsky, 2000; http://fokker.wi.mit.edu/primer3/). 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 (> 0.6 mm of diameter) was placed in a 2-mL tube containing 700 µL CTAB (2.0% of CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM of Tris-HCl, pH=8.0, and 1.0% of polyvinylpyrolidone) plus 2-mercaptoethanol (1 mL of CTAB + 2 µL 2-mercaptoethanol). Two metal beads (2.38 mm) were placed in each vial, followed by two rounds of maceration using a TissueLyser II (Qiagen) for 30 sec at the frequency 25. After maceration, the vials were incubated in a water bath at 65°C for 1 h; the mixture was mixed by inversion at least five times during the incubation. The samples were centrifuged for 10 min at 13750 G. The supernatant (± 500 µL) was transferred to a new 2-mL tube and 2/3 volume (± 340 µL) of isopropanol was added and the mixture gently mixed. The samples were then incubated at -20°C for 30 min. Subsequent centrifugation was conducted at 6740 G for 5 min, and the supernatant was discarded gently to avoid pellet disturbance.

Subsequently, 1 mL of 70% ethanol was added to rinse the pellet for 1 min, and then it was centrifuged at 8803 G for 1 min. The supernatant was discarded and another 1 mL of 70% ethanol was added to the tube, which was maintained overnight in a shaker at room temperature (RT). The supernatant was removed and the pellet was dehydrated in vacuum centrifuge. The pellet was eluted in 50 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA) and RNase (2 µg).

The final 20-µL reaction mixture included 2 µL 10x PCR buffer II, 2 µL of (25 mM) $MgCl_2$, 1 µL of 10mM of each dNTP, 0.3 µL of the forward and reverse primers (5 µM), 0.2 µL *Taq* DNA polymerase (1 U, Bioline), 11.2 µL of water, and 3 µL of DNA template at 10 ng/µL. PCR amplifications were performed using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) with one cycle at 95°C for 5 min, followed by three cycles at 95°C for 30 s, 40 to 55°C (depending on the locus) for 30 s, 72°C for 80 s, 35 cycles at 94°C for 15 s, 40 to 55°C (depending on the locus) for 15 s, and 45 s at 72°C, followed by one cycle of 72°C for 5 min, ending with a holding step at 10°C.

The PCR products were separated using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Product sizes were determined using marker standards and ABI PeakScanner Analysis Software v1.0 (Applied Biosystems Inc.). Each product length (within 2 bp) was considered a different allele. For most loci, alleles differed by increments of 2 bp.

Data Analysis

Isolates were grouped based on the host of origin: EU, PG, SJ, PA, SC, MC, and EG (Table 1). Evidence of structuring by geographic location was also assessed for the EU and PG populations using Analysis of Molecular Variance (AMOVA), based on R_{ST} statistics, on the software GenAlEx 6.4 (Peakall and Smouse, 2006). Number of alleles (N_A), allele frequencies, number of effective alleles (N_E), number of private alleles (*PVA*), and the Shannon's information index (Shannon *I*) were calculated in the GenAlEx 6.4. To examine the influence of undetectable or null alleles in microsatellite loci the software MICROCHECKER 2.2.3 was used (Oosterhout et al., 2004). No evidence of null alleles was detected for any locus. ARLEQUIN 3.5 (Excoffier et al., 2010) was used to calculate the number of genotypes, observed heterozygosity (H_O), expected heterozyogosity (H_E), fixation index (F_{IS}), multilocus genotypic richness, multilocus genotypic evenness, and to test the deviation of the Hardy Weinberg equilibrium (*HWE*). Genetic differentiation among

multilocus genotypes (MGs) from different hosts was calculated through AMOVA with 1000 permutations with R_{ST} that assumes stepwise mutation model in ARLEQUIN 3.5. The theta (θ) statistic (Weir and Cockerham, 1984), which is comparable to Wright's F_{ST}, was calculated among populations pairs using MultiLocus 1.3 (Agapow and Burt, 2001).

Multilocus genotypic diversity was assessed using Stoddart and Taylor's index G (Stoddart and Taylor, 1988). Genotypic diversity was estimated using the technique of rarefaction (Gotelli and Colwell, 2001) to correct for biases due to differences in sample size using an input command implemented in the software R (Ihaka and Gentleman, 1996). To test for random mating among populations, multilocus linkage disequilibrium was analyzed using the index of association (I_A), which is dependent on sample size, and also a measure corrected for the number of loci, r_D in MultiLocus v1.3. Tests of departure from random mating for both I_A and r_D were done with 1000 randomizations.

Principal coordinate analysis (PCA) based on genetic distance (Smouse and Peakall, 1999) between all pairs of MGs was carried on GenAlEx 6.4. A Neighbor-Joining tree based on the stepwise weighted genetic distance measure D_{SW} (Shriver et al., 1995), was constructed and visualized in the software POPTREE2 (Takezaki et al., 2010). The bootstrap values for each tree branch were calculated in the software POPTREE2 using the allele frequency in each loci, with a 1000 bootstrap replications. Multilocus haplotypes and their frequencies were estimated using the Maximum-Likelihood (ML) from the observed data using ELB-algorithm, which attempts to reconstructing the (unknown) gametic phase of multilocus genotypes, in ARLEQUIN 3.5, considering the microsatellite repeat numbers and an unknown gametic phase. The number of repeats of the ten microsatellite loci analysed were determined based on the nucleotide sequence. The relationship among haplotypes was presented by a Median-Joining Network (MJ) generated by the program Network 4.6.0.0 (Bandelt et al., 1999). Network branches correspond to single mutational steps. Median vectors, estimated with a parsimony criterion, were used to represent missing haplotypes. The network was edited using the program Network Published (http://www.fluxus-engineering.com/nwpub.htm). Haplotypes were presented as circles, which are color coded based on host of origin. Circle sizes are proportional to the prevalence of each haplotype in the population.

Results

Ten microsatellite loci (Table 2) were analyzed for 148 *P. psidii* isolates collected on seven hosts from nine states in Brazil (Figure 1; Table 1). All loci were polymorphic and no evidence of null alleles was detected. Two to seven alleles were detected per locus (Table 1) and a total of 25 multilocus genotypes (MGs) were detected. No evidence of subdivision by geographic location was observed within host of origin. For each of both EU and GA populations, 100% of the observed variation was due to differences within individuals and 0% due to differences in geographic origin. As no subdivision by geographic location was observed all subsequent genetic analyses were done clustering isolates by host. MGs were completely associated with host with no identical MGs observed on different hosts. *Eucalyptus* (EU) and *P. guajava* (PG) populations exhibited the largest number of MGs, with 15 and 5, respectively. Within the 15 MGs from EU, only two (EU-13 and EU-9) were frequent (>10%), with frequencies of 14.86 and 10.81%, respectively. Similarly, only two MGs (GA-4 and GA-2) were common (>10%) within the PG population, with frequency of 18.24% and 12.84%, respectively. Although fewer isolates were collected from other hosts, it is especially noteworthy that only single, but completely distinct, MG was observed for each of the following hosts, *P. araca* (PA), *S. jambos* (SJ), *S. cumini* (SC), *M. cauliflora* (MC), and *E. uniflora* (EG).

The EU group had the higher number of alleles per locus. Isolates from EU and MC had the higher number of effective alleles (N_A), and also the highest Shannon information index (I) (Table 3). High levels of observed heterozygosity (H_O) were obtained for all host species. Strong negative values of F_{IS} were detected on all seven host groups, as a result of large differences between H_O and expected heterozygosity (H_E), indicating high rate of asexual reproduction. Private alleles (*PVA*) were observed on most host populations, except the PG population contained no *PVAs*. The largest numbers of *PVA*s were observed on MC and SC populations, with eight and five *PVAs,* respectively. The EG population also exhibited large number of *PVAs* with four (Table 3). The AMOVA, based on R_{ST}, estimated that 4.64% of the observed variance in the gene diversity distribution occurs within individuals and 97.47% of the variance is due to differences between populations, or host of origin ($P > 0.01$; Table 4).

The isolates collected on each host were grouped using (1) a principal coordinate analysis (PCA) plot and (2) a Neighbor-joining method of tree construction. PCA revealed a high degree of genetic differentiation among isolates from distinct hosts. The first two axes

explained 82.86% of the total observed variation, with the first axis explaining 74.23% and the second 8.63%. Five distinct groups were observed: (1) a cluster formed by isolates from EU and SJ, (2) another cluster formed by PG and PA isolates, and three separate groups formed by (3) SC, (4) MC, and (5) EG, respectively (Figure 2). Similarly, the NJ tree, based on Euclidean distance between microsatellite multilocus genotypes, exhibited a cluster formed by EU and SJ isolates, supported by a bootstrap value of 99%. The isolates from PG and PA were also clustered in a fairly distinct group, with a bootstrap value of 80%. Isolates collected on MC, SC, and EG, formed weakly separate clusters (with bootstrap values of 43% and 73%).

Population differentiation was also assessed through R_{ST}, an F_{ST} analogue that assumes stepwise mutation (Slatkin, 1995). Based on the observed R_{ST} values, the host subpopulations differed significantly from each other ($P < 0.05$), with the exception of PG and PA, which were not significantly differentiated (0.187; $P > 0.05$) (Table 5). The high values of R_{ST}, with an overall R_{ST} of 0.975, indicate strong selection by host species on *P. psidii* populations from Brazil. Weir & Cokerhan's Theta (θ) statistics was also used to estimate the degree of differentiation between subpopulations. Theta values vary from 0.0 (no differentiation between populations) to 1.0 (complete differentiation between populations). The EU and SJ populations were the most similar ($\theta = 0.039$; $P < 0.01$), while the PG and EU groups were most differentiated ($\theta = 0.688$) (Table 6). The EU also showed some degree of similarity to PA, SC, and EG (Table 6). The relatively low values of θ also indicated some association between SJ isolates to PA and SC isolates. The significant small value of θ (0.078) indicated a high degree of similarity between PG and PA populations. The remaining populations were significantly differentiated from each other.

The contribution of sexual and asexual reproduction was estimated through linkage disequilibrium across all 10 microsatellite loci on 148 *P. psidii* isolates. The overall linkage disequilibrium was high, with an association index (I_A) of 5.048. Another measure of linkage disequilibrium, r_D, was also elevated (0.596), indicating a high rate of clonal reproduction.

A Median Joining haplotype network (MJ) constructed based on genetic distance between the 27 haplotypes estimated using ARLEQUIN 3.5. The relatively compact network indicated that the *P. psidii* population in Brazil is comprised of a small number of closely related genotypes (Figure 4). However, strong differentiation by host was observed, with private haplotypes found for all host species, except H3 (formed by 38 isolates from EU and 4 isolates from SJ) and H14 (formed by 28 isolates from GA and 2 isolates from PA). The high frequency of private haplotypes also indicates strong selection by host species on Brazilian *P.*

psidii populations. The most prevalent EU haplotypes were H3 (14.19%), H4 (10.14%), H2 (9.80%), and H5 (4.73%). Four haplotypes were also the most common on the PG population, H13 (11.82%), H14 (10.13%), H17 (9.12%), and H15 (6.41%). The median vectors on the Network are a hypothesized (often ancestral) missing haplotypes, which are required to connect the existing haplotypes within the MJ network. The median vector are hypothesized, often ancestral, missing haplotypes, which are required to connect the existing haplotypes within the MJ network. The median vector replaces a missing haplotype to indicate the shortest connection between haplotypes. According to the MJ network, the haplotypes from EU and GA were clustered into two distinct groups. The haplotypes from SJ and SC derived from EU haplotypes. The haplotypes of MC were linked to one haplotype from SJ (H13), and the haplotypes from EG derived from two median vectors responsible for linking the haplotypes cluster of EU and GA.

Discussion

Evidences of strong selection by host and high rate of clonal reproduction in *P. psidii* population from Brazil were revealed by microsatellite multilocus genotypes (MGs). Host species appears to strongly affect the genetic structure of *P. psidii* in Brazil, which means that distinct MGs are uniquely associated with specific hosts across diverse geographic locations. The association of unique MGs to specific host species may indicate that Brazil could represent the evolutionary center of origin of this pathogen, as previously hypothesized (Tommerup et al., 2003; Glen et al., 2007). The large numbers of private alleles associated with isolates collected on a given host species also indicate strong host specialization. Multiple events of *P. psidii* introduction into Brazil might be an alternative explanation for such strong association of specific pathogen genotypes within each host species. In this unlikely scenario, the hypothesy is that the fungus is not native from Brazil, and that distinct events of pathogen introduction occurred in different times. However, based on the consistent chronology of *P. psidii* descriptions worldwide it is reasonable to propose that the fungus is native from Brazil (Glen et al., 2007; Loope, 2010). The AMOVA, based on R_{ST}, supports the hypothesis of host selection in *P. psidii*, estimating that 97% of the observed variance is due to differences among isolates from different host species (Table 4). The subdivision by host on the pathogen population from Brazil was also shown through PCA analysis, indicating high degree of genetic differentiation among isolates collected on different host species. Five major groups were revealed on the PCA plot, the first formed by EU and SJ isolates, the

second by PG and PA isolates, and three weakly separate groups formed by isolates from SC, MC, and EG, respectively. A very similar pattern was observed on the NJ tree, with the population clustered on five major groups (Figure 3), and in the same way as in the PCA plot the isolates from MC were considered the most distinct. Population structure was also analyzed by R_{ST}, an F_{ST} analogue that assumes stepwise mutation (Stalkin, 1995). Even though some studies indicate weakness of R_{ST} and its analogues, to detect population structure, suggesting that none of these statistics can be used to infer population differentiation or similarity (Hedrik, 2005; Jost, 2008), we used the R_{ST} method as an additional tool to support our previously statements on population structure. Based on R_{ST}, the isolates collected on different host species differed significantly from each other ($P < 0.05$; Table 5), with the exception of PG and PA, which were not significantly differentiated ($P > 0.05$). The overall R_{ST} value was 0.975, might indicate strong selection by host species on *P. psidii*. In addition, Weir & Cokerhan's θ statistics was also used to estimate the population structure based on host of origin. According to θ, EU population was extremely similar to the SJ population ($\theta =$ 0.039). The low θ value observed between PG and PA (0.078) indicated that the isolates from those hosts did not differ. The average θ value among populations was 0.548, indicating differentiation among the populations.

Our genetic data based on the microsatellite MGs indicating host selection on *P. psidii* population from Brazil is strongly supported by previously studies of *P. psidii* virulence phenotypes, that also indicate a high degree of host specialization (Aparecido *et al.* 2003, Castro *et al.* 1983, Coelho *et al.* 2001, Coutinho and Figueiredo 1984, Ferreira 1981, Joffily 1944, Maclachlan 1938). Existence of *P. psidii* races or biotypes were also reported (Xavier et al., 2002; Graca et al., 2011); although in comparison with other rusts such as those of cereal crops (Goyeau et al., 2007), very little is known about host specialization on *P. psidii*. Several cross inoculations were carried out to characterize *P. psidii* isolates phenotype on different host species. In Jamaica, *P. psidii* isolates collected on *Pimento* spp. and *Syzygium* spp. did not infected *P. guajava*, and the isolate collected on *Pimento* spp. were also unable to infect *S. jambos* (MacLachlan 1938). Rust isolates collected on infected *M. quinquenervia* plants and on *Pimenta dioica* in Florida, USA, did not infect *S. jambos* (Rayachhetry *et al*. 2001). In Brazil, isolates collected on *P. guajava* did not infect *Eucalyptus*, and isolates collected on *Eucalyptus* did not infect *P. guajava* (Ferreira 1983). Three groups of physiological specialization were observed through *P. psidii* cross-inoculation studies, one compatible with *E. grandis* and *S. jambos*, another with *E. grandis* and *P. guajava*, and the last only with *P.*

guajava (Coelho *et al*. 2001). In the current study, we also observed phenotypic variation among *P. psidii* isolates from different hosts in Brazil, with some incompatible interactions on cross inoculations. Isolates collected on PG commonly cause HR and induced strong defoliation on SJ cuttings, the same symptoms were observed when SJ cuttings were inoculated with PA isolates (unpublished data). The phenotypic variation observed on *P. psidii* populations around the world corroborates with our molecular data based on *P. psidii* microsatellite genotypes, indicating strong effect of host selection on populations of this rust pathogen.

In addition to the strong evidence of *P. psidii* specialization according to host species, our data provide strong evidence of high rate of clonal reproduction within *P. psidii* populations from Brazil. The high proportion of repeated MGs observed within each host, combined with the high values of I_A and r_D (indicating significant linkage disequilibrium), and the significant low values of F_{IS}, indicates high asexual reproduction rate in the Brazilian rust population. This rust is supposed to be autoecious with an incomplete lifecycle (Glen et al., 2007). All rust stages are formed on the same Myrtaceous host, although the spermogonia (or picnia) stage has never been reported. *Puccinia psidii* was proposed to be heteroecious with an unknown aecial host (Simpson *et al*. 2006). However, reported infections of *S. jambos* by rust basidiospores (Figueiredo 2001) do not support the hypothesis of an alternate host. Detailed cytological studies are required to find out if the basidiospores really infect the host tissue and if so, how it happens and how the dikaryotization from haploid basidiospores to dikaryotic aeciospores occurs. However, even the direct observation of sexual structures cannot provide an accurate estimate of the proportion of clonal or sexual reproduction in natural populations of plant pathogens. Rapidly mutating molecular markers, such as microsatellites, provide the best estimates of reproductive behavior in plant pathogen populations (Taylor et al., 1999; Halkett et al., 2005). The high levels of heterozygotes (Birky, 1996; De Meeus and Balloux, 2004; Halkett et al., 2005) are the supporting evidence for clonal reproduction on *P. psidii* population in Brazil. The detection and estimation of clonal reproduction rate using microsatellite markers has been demonstrated on rust populations infecting cereal crops (Goyeau et al., 2007; Ordoñez et al., 2010), but no such studies have been previously conducted with rusts on forest species.

The haplotype MJ-Network also supported the hypothesis of host selection and clonal reproduction on *P. psidii* population from Brazil. The compact MJ-network suggests that *P. psidii* population in Brazil is comprised by a small number of closely related genotypes. The

high frequency of private haplotypes within each host also indicates strong selection by host species. The MJ-network, clustered the haplotypes from EU and GA into two distinct groups. The most frequent haplotypes on the MJ-network H2, H3, and H4 from EU, and H14, H16, H17, and H18, from PG, may correspond to the founding MGs in the population. Genetic diversity on these hosts was also higher when compared to isolates collected on other hosts, as expected due to the larger number of samples. However, the fairly distinct haplotypes found on hosts with lower number of isolates (SJ, PA, SC, MC, and EG) across different geographic locations take us to conclude that the observed subdivision based on host species was not a result of small sample sizes.

Host species provides strong selection pressure on *P. psidii* populations from Brazil, regardless of geographic location, and no evidence of sexual reproduction was observed. The high rate of clonal reproduction is strongly supported by significant high values of observed heterozygosity. The moderate degree of genetic diversity observed among isolates from EU and PG is probably generated by rare mutation events, which were probably responsible for the rust isolates adaptation to unique host species. Although sample numbers are low, several host species in Brazil appear to be infected by a unique genotype of *P. psidii* that only occurs on one host species. This perhaps represents recent host shifts by *P. psidii* in South America. Furthermore, the existence of host-specific genotypes of *P. psidii* in Brazil couldindicate that *P. psidii* may be evolving toward speciation by host. Perhaps, such strong selection by host species might also indicate occurence of cryptic species within the so called *P. psidii* complex. This hypothesis is currently under study using phylogenetic approaches, as a joint project of Federal University of Viçosa, USDA-Forest Service, Washington State University, University of Hawaii at Manoa, and Universty of Tasmania.

Figure 1. Map of the *Puccinia psidii* localities sampled in Brazil. Black dot represents the location of each isolate based on GPS coordinates.

Table 1. Sources of *Puccinia psidii* isolates collected on Myrtaceae species in nine Brazilian states.

Host	State	No. of isolates
Eucalyptus spp. (EU)	Bahia	18
	Espirito Santo	16
	Minas Gerais	2
	Parana	3
	Rio de Janeiro	1
	Santa Catarina	7
	Sao Paulo	22
	Uruguay	1
Psidium guajava (PG)	Espirito Santo	12
	Minas Gerais	32
	Rio de Janeiro	10
	Rio Grande do Sul	4
	Sao Paulo	5
Syzigium jambos (SJ)	Mato Grosso do Sul	1
	Minas Gerais	2
	Rio de Janeiro	1
Psidium araca (PA)	Minas Gerais	2
Syzigium cumini (SC)	Rio de Janeiro	2
	Sao Paulo	2
Myrciaria cauliflora (MC)	Minas Gerais	1
	Rio de Janeiro	1
	Sao Paulo	1
Eugenia uniflora (EG)	Minas Gerais	2
Total		148

Table 2. Primer sequences and characteristics of 10 microsatellite markers used on to characterize *Puccinia psidii* populations in Brazil (adapted from Zhong et al., 2008).

Locus	GenBank accession no.	Primer sequence (5' - 3')	gc%	Repeat motif	No. of alleles	Size range
PpSSR012	EF523501	F: TTCAATCCCCATAAGGCTTTC R: AAATCCTGAGTCTTCTTCCCC	42.86 47.62	$(AG)_9$	5	229 - 239
PpSSR014	EF523502	F: TTCGACATCCAACGCTCTCAT R: AAAGGCTAAGTGAATGGGCA	47.62 45	$(AG)_{13}$	4	206-214
PpSSR018	EF523503	F: AGCCTTCTCTCTCCTCCGTTA R: TCAGGAAGGACAAGACCAAGT	52.38 47.62	$(AG)_9$	4	162-172
*PpSSR022**	EF523504	F: CCTTTAGGCTGTGGTTTCCA R: GCCCACTCTGTCAAGAGGAA	50 55	$(AG)_{12}$	6	147-159
*PpSSR087**	EF523507	F: AAGAACGTGAACGGGAATGA R: GAAATGCCAGACGAAGGGTA	45 50	$(AG)_{14} + G_6 + A_9$	7	154-178
*PpSSR102**	EF523508	F: TGACTTTAATCATCTTCAAAACCAA R: ACCAATCCCCTTCCTTCATC	28 50	$T_7 + (AG)_{22} + T_7$	2	140-142
*PpSSR146**	EF523510	F: TTGGTAAAGAGGAGGGGATTC R: TCAGCACCAACCATTACCTTC	47.62 47.62	$(AG)_{73}$	7	205-249
PpSSR161	EF523511	F: TCGAGGGGTCTCAGTTTTCA R: GAGATCTATCGGACCAACGAA	50 47.62	$(AG)_{25}$	5	272-292
*PpSSR178**	EF523512	F: TCGTGTGCATGTGTGTATCG R: GCCTTGGGTGCACACTTTAT	50 50	$(AG)_{62}$	7	167-293
*PpSSR195**	EF523513	F: GAACGAACCCAAACTTTCCA R: GGAAAGGAATGAGATTGAACACA	45 39.13	$(AG)_{18}$	3	208-212

***** New set of primers designed on the software Primer3 based on the *P. psidii* microsatellite sequences deposited in the GenBank (Zhong et al., 2008).

Table 3. Overall population genetics statistics of *Puccinia psidii* isolates collected on different hosts and geographic locations in Brazil.

Parameters[a]	**Host**[b]						
	Eucalyptus	***S. jambos***	***P. guajava***	***P. araca***	***S. cumini***	***M. cauliflora***	***E. uniflora***
No. of alleles	2.100 (0.314)	1.600 (0.163)	1.700 (0.300)	1.600 (0.163)	1.700 (0.153)	1.800 (0.133)	1.300 (0.153)
No. of effective alleles	1.754 (0.191)	1.600 (0.163)	1.625 (0.297)	1.600 (0.163)	1.700 (0.153)	1.800 (0.133)	1.300 (0.153)
Shannon index *I*	0.536 (0.127)	0.416 (0.133)	0.384 (0.150)	0.416 (0.133)	0.485 (0.106)	0.555 (0.092)	0.208 (0.106)
H_O	0.630 (0.147)	0.600 (0.163)	0.425 (0.158)	0.600 (0.163)	0.700 (0.153)	0.800 (0.133)	0.300 (0.153)
H_E	0.354 (0.080)	0.300 (0.082)	0.247 (0.091)	0.300 (0.082)	0.350 (0.076)	0.400 (0.067)	0.150 (0.076)
F_{IS}	– 0.767 (0.082)	– 1.000 (0.000)	– 0.697 (0.133)	– 1.000 (0.000)	– 1.000 (0.000)	– 1.000 (0.000)	– 1.000 (0.000)
No. of haplotypes	13	2	6	2	2	2	2
PVA	2	1	0	1	5	8	4

[a] H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = fixation index; and *PVA* = number of private alleles.
[b] Numbers in parentheses = standard error.

Table 4. Analysis of molecular variance (AMOVA), based on R_{ST} statistics, among *Puccinia psidii* isolates collected on different host species and geographic locations in Brazil.

Source	**d.f.**	**Sum of squares**	**Percentage of variation**	***P***	**Fixation index**
Among hosts	6	156794.49	97.47	0.001	0.974
Among individuals within hosts	141	553.37	-2.11	1.000	-0.831
Within individuals	148	6296.50	4.64	0.001	0.954
Total	295	163644.36	100		

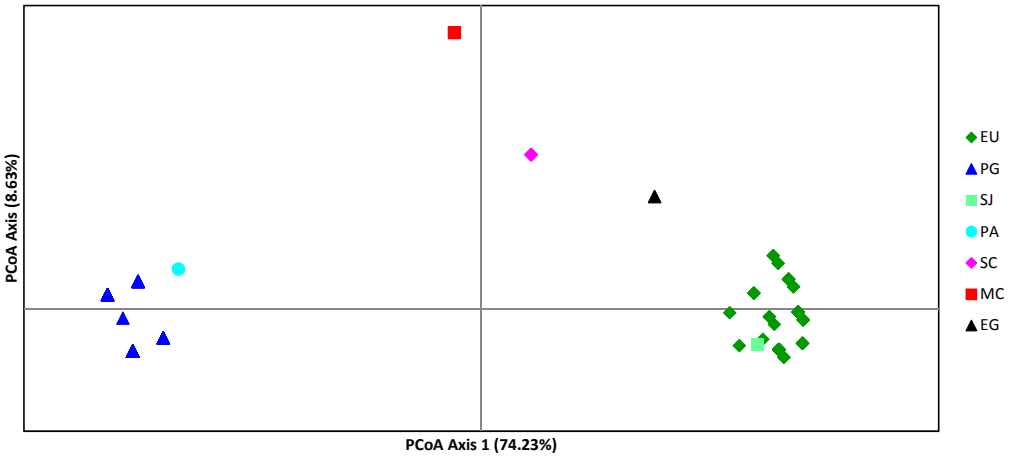

Figure 2. Principal coordinate analysis (PCA) plot of microsatellite multilocus genotypes (MGs) of 148 *Puccinia psidii* isolates collected in different hosts in Brazil based on the genetic distance between MGs. The first two axes explain 82.86% of the observed variation. (EU, *Eucalyptus*; PG, *Psidium guajava*; SJ, *Syzigium jambos*; PA, *Psidium araca*; SC, *Syzigium cumini*; MC, *Myrciaria cauliflora*; EG, *Eugenia uniflora*).

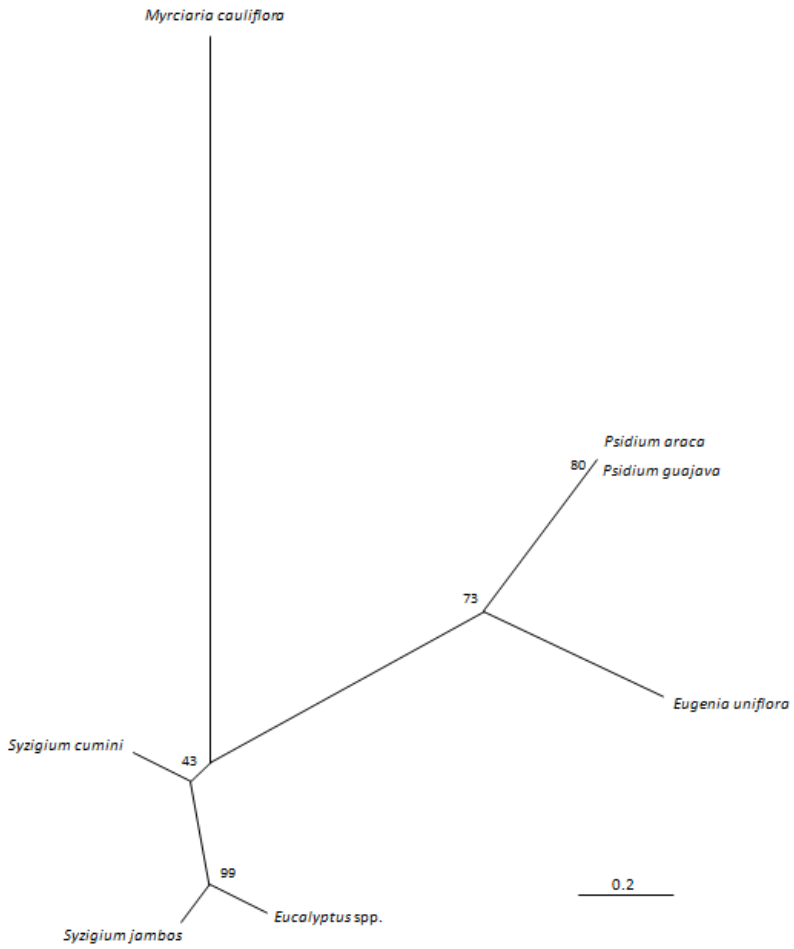

Figure 3. Neighbor joining tree of the multilocus microsatellite genotypes showing the relationship between *Puccinia psidii* isolates from seven hosts. Bootstrap values based on 1000 replications are shown. Branch length corresponds to genetic distance between groups.

Table 5. *Puccinia psidii* pairwise population R_{ST} estimated between isolate hosts of origin. R_{ST} values below diagonal. Probability values based on 999 permutations are shown above diagonal.

Host	Host of origin						
	Eucalyptus	***P. guajava***	***S. jambos***	***P. araca***	***S. cumini***	***M. cauliflora***	***E. uniflora***
***Eucalyptus* spp.**	0.000	0.001	0.001	0.001	0.001	0.001	0.001
Psidium guajava	0.981	0.000	0.001	0.059	0.001	0.001	0.001
Syzigium jambos	0.612	0.975	0.000	0.002	0.041	0.001	0.001
P. araca	0.985	0.187*	0.939	0.000	0.001	0.004	0.010
Syzigium cumini	0.636	0.970	0.278	0.955	0.000	0.001	0.001
Myrciaria cauliflora	0.946	0.987	0.715	0.983	0.874	0.000	0.002
Eugenia uniflora	0.980	0.592	0.929	0.770	0.948	0.985	0.000

* All values significant at $P < 0.05$, except those indicated with *, where $P > 0.05$.

Table 6. Likelihood estimates of Weir & Cockerham's theta (θ) between all pairs of *Puccinia psidii* populations from seven hosts in Brazil. Theta θ values below diagonal. Probability values based on 999 permutations are shown above diagonal.

	Host of origin						
Host	***Eucalyptus***	***S. jambos***	***P. guajava***	***P. araca***	***S. cumini***	***M. cauliflora***	***E. uniflora***
***Eucalyptus* spp.**	0.000	0.001	0.001	0.001	0.001	0.001	0.001
Psidium guajava	0.039*	0.000	0.001	0.068	0.025	0.036	0.071
Syzigium jambos	0.565	0.601	0.000	0.001	0.002	0.001	0.001
P. araca	0.490*	0.480*	0.078*	0.000	0.058	0.095	0.331
Syzigium cumini	0.469*	0.469*	0.613	0.467	0.000	0.026	0.059
Myrciaria cauliflora	0.573	0.551	0.656	0.459	0.516	0.000	0.001
Eugenia uniflora	0.460*	0.495	0.688	0.600	0.576	0.604	0.000

*$\theta < 0.5$ indicate significantly low differentiation between populations.

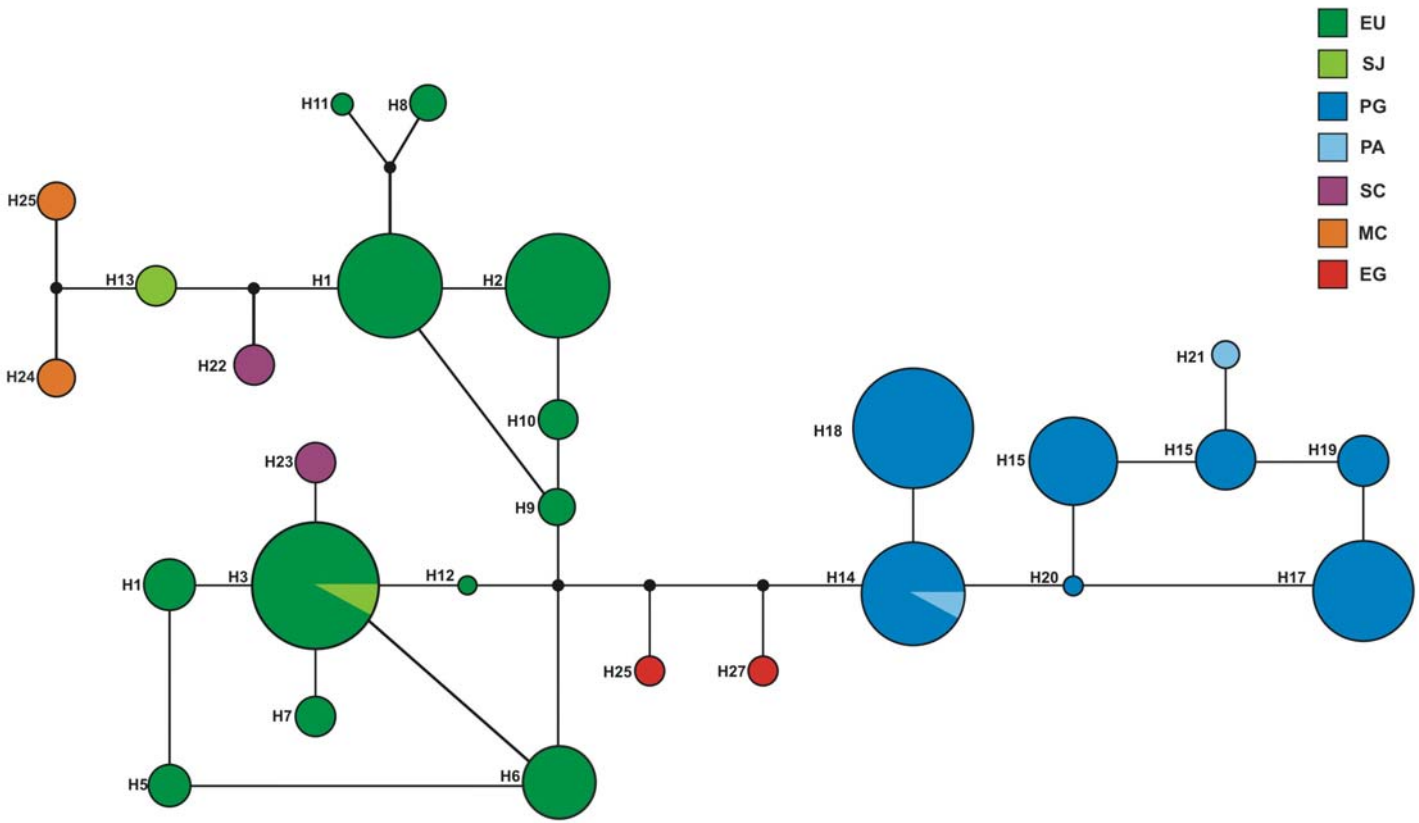

Figure 4. Median-joining (MJ) network of *Puccinia psidii* on different hosts (EU, *Eucalyptus*; SJ, *Syzigium jambos*; PG, *Psidium guajava*; PA, *Psidium araca*; SC, *Syzigium cumini*; MC, *Myrciaria cauliflora*; EG, *Eugenia uniflora*), based on 10 microsatellite markers. Missing or unsampled are represented by median vectors (black dots). Sizes of haplotypes circles indicate haplotypes frequencies. The haplotypes from different host populations are color coded.

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3. ARTICLE THREE

Tracking down worldwide *Puccinia psidii* dispersal

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Abstract

Fast-evolving microsatellite markers were used to determine genetic relationships among rust populations from South America, California and Hawaii. Such genetic markers allow inferences about the potential sources of pathogen introduction. The hypotheses are that *P. psidii* was introduced into Hawaii through California by trade of rust infected myrtaceous plants, and that *P. psidii* populations from South America are distinct from the rust populations that became estabilished in California and Hawaii. The eight microsatellite loci analyzed revealed 14 multilocus genotypes (MGs) within the 221 *P. psidii* isolates. Isolates collected on different hosts in South America presented distinct MGs. In contrast, all rust isolates collected on nine myrtaceous hosts in the Hawaiian Islands were composed of only a single, unique MG, suggesting a recent introduction of a single *P. psidii* genotype into the

Hawaiian Islands. The same unique MG observed in Hawaii, was also detected on isolates from two different hosts in California, indicating a common origin of the rust genotype found in Hawaii and California. The MG comprising all isolates from Hawaii and California is distinct from the MGs found in South America so far, suggesting that the Hawaiian and Californian isolates did not come directly from South America. Isolates from Florida, Central America, and the Caribbean must be analyzed to better understand potential relationships with pathogen dispersion to Hawaii.

Introduction

Puccinia psidii causes rust disease on many host species in the Myrtaceae (Farr and Rossman, 2010). First reported in 1884 on guava in Southern Brazil (Maclachlan, 1938), the rust has since been detected on several myrtaceous in South America (Ferreira, 1981; Lindquist, 1982; Kern et al., 1933; Kobayashi, 1984; Chardon and Toro, 1934), Central America (Bernt, 2004; Perdomo-Sanches and Piepenbring, 2008), Caribbean (Spaulding, 1961; Baker and Dale, 1948; Kern et al., 1933; Dale, 1955; Stevenson, 1975), Mexico (Gallegos and Cummins, 1981), USA: in Florida (Rayachhertry et al., 1997), California (Mellano, 2006), and Hawaii (Uchida et al., 2006). More recently, *P. psidii* was reported in Japan infecting *M. polymorpha* (Kawanishi et al., 2009). An unconfirmed report of this rust on *Eucalyptus camaldulensis* in Taiwan also exists (Wang, 1992; Glen et al., 2007). Of special note is that a rust was found infecting Myrtaceae species in Australia, the fungus was reported as *Uredo rangelii*, based on the tonsure found on the urediniospores surface. However, DNA sequence data did not differentiate that rust from *P. psidii* (Carnegie et al., 2010), and the same tonsure patch, observed on the Australian urediniospores, was also observed, using scanning electron microscope, on rust urediniospores collected from several host species in Brazil (A.C. Alfenas and R.N. Graça unpublished data). Population genetics studies are needed to elucidate the disease etiology in Australia.

This rust fungus is expanding its geographic distribution, and based on the potential damage to diverse myrtaceous species, *P.psidii* is considered a major potential threat to the Myrtaceae in Southeast Asia and surrounding Islands, Australia, and South Africa (Ciesla et al., 1996; Coutinho et al., 1998; Tommerup et al., 2003; Glen et al., 2007; Carnegie et al., 2010). Many myrtaceous species from Australia, Indonesia, Papua New Guinea, Thailand and Vietnam were tested for rust resistance in Brazil, and the vast majority was classified as highly susceptible (Zauza et al., 2010). A similar study is underway to access the degree of

susceptibility of Hawaiian native species to *P. psidii* isolates collected on different host species in Brazil.

In Hawaii, *P. psidii* infects several native and introduced Myrtaceae species including, an endemic tree species known as ‘ohi’a (*Metrosideros* spp.), the dominant tree species in Hawaii’s remnant native forests, the endangered species Nioi (*Eugenia koolauensis*), as well as the invasive species Rose Apple (*Syzigium jambos*) (Uchida et al., 2006; Loope, 2010). The rust was supposedly introduced in Hawaii, through rust infected ornamental plants from California (Uchida et al., 2006; Loope, 2010), where *P. psidii* had been previously reported (Mellano, 2006). This hypothesis is supported by the Hawaiian department of Agriculture which intercepted in 2006 and 2007 *P. psidii* infected plant materials shipped from California (Loope, 2010). However, there is no conclusive proof that *P. psidii* was introduced to the Hawaiian Islands from California. Of present concern is the potential introduction of new pathogen genotypes into Hawaii, because more aggressive isolates could potentially cause even greater damage to Hawaiian native forests.

Population genetic studies based on microsatellite multilocus genotypes indicated strong selection by host species or maybe, multiple events of rust spores introductions in Brazil (Graça et al., 2011). However, information is needed to understand the genetic relationship between *P. psidii* populations from South America (Brazil, Uruguay, and Paraguay) and the rust population estabilished in California and Hawaii. Understanding the global genetic structure of *P. psidii* populations is essential for inferences about migratory routes and sources of introductions.

A primary objective of this study is to track the dispersal route of *P. psidii* into Hawaii, as well to provide baseline information essential to identify rust genotypes that pose threats to global Myrtaceae populations. This information can be used to help prevent the introduction of critical rust genotypes into new regions, and increase the efficacy in selecting rust-resistant host individuals. In this study microsatellite loci were analyzed to determine the genetic relationship among rust isolates from South America, California and Hawaii. The hypotheses are that *P. psidii* was introduced in Hawaii through California by trade of rust infected myrtaceous plants, and that *P. psidii* populations from South America are differentiated from the rust populations estabilished in California and Hawaii, as result of geographic isolation.

Material and Methods

Sampling

In addition to the 148 *P. psidii* isolates from Brazil previously tested for microsatellite multilocus genotypes (Graca et al., 2011), we collected 15 new isolates on different Myrtaceae hosts in Brazil, one isolate in Paraguay, five isolates in Uruguay, and in USA: two isolates in California and 50 in Hawaii (Table 1). A total of 221 single uredinial isolates were collected from March of 2008 to December of 2010. All survey points were georeferenced using a GPS. Each isolate was comprised of three distinct single uredinial pustules (> 0.6 mm of diameter) collected from each host and location. The single pustules were placed separately in 2-mL Eppendorf® vials, which were stored at -80°C prior to DNA extraction. One pustule from each isolate was used for DNA extractions, and the remaining two were kept at -80°C.

Microsatellite genotyping

Eight microsatellite loci were scored for 221 *P. psidii* isolates. These loci are *PpSSR*012, *PpSSR*014, *PpSSR*018, *PpSSR*022, *PpSSR*087, *PpSSR*102, *PpSSR*146 and *PpSSR*195 (Zhong et al., 2008; Graca et al., 2011). Genomic DNA was extracted directly from a single *P. psidii* pustule (fungus + host tissue) using the modified CTAB-based protocol (Doyle and Doyle, 1987; Graça et al., 2011). The PCR was conducted as 20 µL reaction mixtures that contained 10x PCR buffer II, 2 µL of (25 mM) $MgCl_2$, 1 µM of 10mM of each dNTP, 0.3 µL of the forward and reverse primers (5 µM), 0.2 µL *Taq* DNA polymerase (1 U, Bioline), and 3 µL of DNA template at 10 ng/µL. PCR amplifications were performed using a MyCycler Thermal Cycler (Bio-Rad) with one cycle at 95°C for 5 min, followed by three cycles at 95°C for 30 s, 40 to 55°C (depending on the locus) for 30 s, 72°C for 80 s, 35 cycles at 94°C for 15 s, 40 to 55°C (depending of the locus) for 15 s, and 45 s at 72°C, followed by one cycle of 72°C for 5 min, ending with a holding step at 10°C.

The PCR products were separated using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc.). Product sizes were determined using marker standards and ABI PeakScanner Analysis Software v1.0 (Applied Biosystems Inc., Foster City, CA). Each product length (within 2 bp) was considered a different allele. For most loci, alleles differed by increments of 2 bp.

Data analysis

The newly collected rust isolates from Brazil (Table 1) were clustered in groups based on the host of origin (EU, *Eucalyptus* spp.; PG, *Psidium guajava*; SJ, *Syzigium jambos*; PA, *P. araca*; SC, *S. cumini*; MC, *Myrciaria cauliflora*; and EG, *Eugenia uniflora*), together with the 148 isolates previously analyzed in a *P. psidii* population genetic study (Graça et al., 2011). Number of alleles (N_A), allele frequencies, number of effective alleles (N_E), number of private alleles (*PVA*), and the Shannon's information index were calculated in the GenAlEx 6.4. To examine the influence of undetectable or null alleles in microsatellite loci the software MICROCHECKER 2.2.3 was used (Oosterhout et al., 2004). ARLEQUIN 3.5 (Excoffier et al., 2010) was used to test the deviation of the Hardy-Weinberg equilibrium (*HWE*) and calculate the number of genotypes, observed heterozygosity (H_O), expected heterozyogosity (H_E), fixation index (F_{IS}), multilocus genotypic richness, and multilocus genotypic evenness. Genetic differentiation among multilocus genotypes (MGs) from different hosts was calculated through AMOVA with 1000 permutations with R_{ST} that assumes stepwise mutation model in ARLEQUIN 3.5. The theta statistic (θ) (Weir and Cockerham's, 1984), which is comparable to Wright's F_{ST}, was calculated among populations pairs using MultiLocus 1.3 (Agapow and Burt, 2001).

Multilocus genotypic diversity was assessed using Stoddart and Taylor's index G (Stoddart and Taylor, 1988). Genotypic diversity was also rarefied to correct the bias due to differences in sample size using an input command implemented in the software R (Ihaka and Gentleman, 1996). To test for random mating among populations, multilocus linkage disequilibrium was analyzed using the index of association (I_A), which is dependent of sample size, and also a measure corrected for the number of loci, r_D in MultiLocus v1.3 (Agapow and Burt, 2001). Tests of departure from random mating for both I_A and r_D were done with 1000 randomizations.

Principal coordinate analysis (PCA) based on a pairwise genetic distance matrix (Smouse and Peakall, 1999) between all pairs of MGs was carried on GenAlEx 6.4. A Neighbor-Joining tree based on the stepwise weighted genetic distance measure D_{SW} (Shriver et al., 1995), was constructed and visualized in the software POPTREE2 (Takezaki et al., 2010). The bootstrap values for each tree branch were calculated in the software POPTREE2 using the allele frequency for each loci, with a 1000 bootstrap replication. Multilocus haplotypes and their frequencies were estimated using the Maximum-Likelihood (ML) from the observed data using ELB-algorithm, which reconstruct the, unknown, gametic phase of

multilocus genotypes, in ARLEQUIN 3.5, considering the microsatellite repeat numbers and an unknown gametic phase. The relationship among haplotypes was presented by a Median-Joining Network (MJ) generated by the program Network 4.6.0.0 (Bandelt et al., 1999). Network branches correspond to single mutational steps. Median vectors, estimated with a parsimony criterion, were used to represent missing haplotypes. The network was edited using the program Network Published (http://www.fluxus-engineering.com/nwpub.htm). Haplotypes were presented as circles, which are color coded based on host of origin. Circle sizes are proportional to the prevalence of each haplotype in the population.

Results

The microsatellite markers revealed four to eight alleles depending on the rust isolate group and locus (average 5 alleles per host) (Table 2). All loci were polymorphic with no evidence of null alleles. The five *P. psidii* isolates from Uruguay (Table 1) were collected on *Eucalyptus grandis*, and all of these isolates had the same multilocus genotype (MG) that clustered in a separate group (UR). The rust collected on *P. guajava* in Paraguay was designated to a distinct group (PY). A single, unique MG was detected in all 50 isolates collected on nine different Myrtaceae hosts in four Hawaiian Islands. As no genetic difference was observed among all Hawaiian isolates, they were placed in a distinct cluster (HI). The isolate collected on *Melaleuca lecodendron* and the isolate collected in *Myrtus communis* in California formed the last group (CA), with an unique MG, identical to the MG found in Hawaii. A total of 14 MGs were detected across all 221 *P. psidii* isolates, seven MGs were observed infecting *Eucalyptus* spp. (EU). The other hosts had just one MG each, regardless of geographic location. Isolates collected on different hosts in South America had distinct MGs, as exemplified by isolates collected on *Eucalyptus grandis* in Uruguay (UR) having an identical MG in the EU population in Brazil, and the *P. guajava* isolate from Paraguay (PY) possessed the same MG that was observed on *P. guajava* isolates from Brazil (PG). In contrast with the South American rust groups, a single, unique MG was identified from all 50 isolates collected from nine hosts on four Hawaiian Islands (Maui, Oahu, Kauai, and Big Island). The same MG, identical to the MG found in Hawaii, was also detected on the two isolates from different hosts in California.

The EU group had the highest number of alleles per locus, followed by MC, HI, and CA groups, which had an equal mean number of alleles (Table 3). However, higher number of effective alleles (N_A) was observed on MC, HI, and CA groups. The isolates from those three

groups also had the highest Shannon information index (*I*) (Table 3). High levels of observed heterozygosity (H_O) were obtained for all *P. psidii* groups. The highest H_O values were observed on MC, HI and CA. Strong negative values of F_{IS} were detected over all 11 rust groups, as a result of large differences between H_O and expected heterozygosity (H_E), indicating a high rate of asexual reproduction within each group. Private alleles (*PVA*) were observed in most groups except PG and PY, which had no *PVAs*. The highest number of *PVA*s were observed in the MC group, with five *PVAs*. When the HI and CA groups were combined together, because they had the same unique MG, four *PVAs* were observed. In addition, the EG population also exhibited three *PVAs* (Table 3). The AMOVA, based on R_{ST}, estimated that 13% of the observed variance in the gene diversity distribution occurs within individuals and 94% of the variance is due to differences among the 11 rust groups (Table 4).

The isolates were grouped using a principal coordinates analysis (PCA), and by the neighbor-joining method of tree construction. The PCA plot revealed high degree of genetic differentiation among isolates from the 11 groups. The first two axes explained 89% of the total observed variation, with the first axis explaining 50% and the second 39%. Based on the PCA, five distinct groups were formed: (1) a cluster formed by isolates from EU, SJ, UR, and EG; (2) another cluster formed by PG, PY, and PA isolates, (3) a group formed by the HI and CA isolates, (4) a separate group with SC isolates, and (5) a separate group with MC isolates (Figure 2). According to the PCA, the SC group is the South American group that is most closely related to the HI and CA groups. Similarly, the NJ tree, based on Euclidean distance between microsatellite multilocus genotypes, exhibited a cluster formed by EU and UR isolates, supported by a bootstrap value of 64%. EU and UR formed a separate major group that contained the SJ isolates (bootstrap = 94%). The isolates from PG and PY were grouped together and formed a cluster with the PA isolates (bootstrap = 85%). A distinct cluster was formed by the HI and CA isolates. The MC group was the South American group that appeared most similar to the HI and CA group, supported by a bootstrap value of 78%. Isolates collected on SJ, SC, and EG formed separate clusters (Figure 3).

The differentiation among *P. psidii* groups was also assessed using R_{ST} statistics, an F_{ST} analogue that assumes stepwise mutation (Slatkin, 1995), shown in Table 5. Based on the observed R_{ST} values, the EU group did not differ from UR group (0.000; $P < 0.05$). The SJ population had some similarity with PA, SC, EG, and PY groups (Table 5). Based on R_{ST} there was no difference between PG and PA groups (0.099; $P < 0.05$), and PG was also quite similar to PY (0.000; $P > 0.05$). The SC and PY group were also undistinguishable (0.280; *P*

< 0.05). As expected, HI and CA did not differ based on R_{ST} (0.000; $P < 0.05$). The high values of R_{ST}, with an overall R_{ST} of 0.936, indicate strong differentiation among the 11 rust groups analyzed. Weir & Cokerhan's Theta (θ) statistics was also used to estimate the degree of differentiation between groups (Table 6). The EU group was very similar to SJ and UR (θ = 0.062 and -0.049, respectively), while the EU and PG groups were highly differentiated (θ = 0.566). The SJ and UR groups were also closely related (θ = -0.016). Each of the following group pairs were also very similar, PG and PY (θ = -0.148), PA and PY (θ = -0.107), and HI and CA (θ = -0.149).

The contribution of sexual and asexual reproduction was estimated through linkage disequilibrium based on all eight microsatellite loci across the 221 *P. psidii* isolates. The overall linkage disequilibrium was high, with an association index (I_A) of 3.942. Another measure of linkage disequilibrium, r_D, was also elevated, 0.597, indicating a high rate of clonal reproduction. The hypothesis of asexual reproduction is strongly supported by high values of H_O. The presence of a single, unique MG on all *P. psidii* groups, except EU with 7 MGs, also indicates absence of sexual reproduction, especially on non-*Eucalyptus* hosts.

The relationship among rust isolates was also estimated by a Median Joining haplotype network (MJ), based on the genetic distance between the 20 haplotypes. Strong differentiation by host was observed among South American groups, with private haplotypes found on all host species, regardless geographic location (Figure 4). Only six of 20 haplotypes were common on more than one rust group. The haplotype H1 with 73 EU isolates and five isolates from UR; H4 with 45 EU isolates, five UR isolates and four isolates of SJ; H10 formed by 63 PG isolates, three PA and two PY isolates; haplotype H11 with 63 PG and two PY isolates; and the Hawaiian an Californian haplotypes, H19 and H20, which comprises all 50 HI isolates and the two CA isolates, each. The high frequency of private haplotypes within each *P. psidii* group in South America mightindicate strong selection by host species on this continent, or could also be an indicative of multiple events of *P. psidii* introductions into South America The detection of only two haplotypes, H19 and H20, in Hawaii across nine Myrtaceae hosts, indicate a recent introduction and establishment of a single rust genotype into Hawaii. The same two haplotypes were also observed in the CA group, indicating common origin of the rust genotype in Hawaii and California. The more frequent haplotypes on the entire network were H1 (18%), H10 (15%), H11 (15%), H4 (12%), H19 and H20 with 12% each. The median vectors on the Network are a hypothesized (often ancestral) missing haplotypes, which are required to connect the existing haplotypes within the MJ network. The median

vector replaces a missing haplotype to indicate the shortest connection between haplotypes. According to the MJ network, three distinct groups were formed, the first with isolates from EU, SJ, and UR, the second with isolates from GA, PY, and PA, and the last group with isolates from HI and CA. The SC haplotype H14 is most closely related to the EU group; however the other SC haplotype, H13 was related to the PG and PA groups. The two MC haplotypes were the most distinct among all South American haplotypes, and were more closely related to the haplotypes from HI and CA. The haplotypes H19 and H20 formed by HI and CA isolates were the most distinct haplotypes on the entire network. Similar to that observe with the PCA plot and the NJ-dendrogram, the MC group was the South American group that was most closely related to the HI and CA group; however it is especially noteworthy that no South American haplotype was identical to the haplotypes found in Hawaii and California.

Discussion

The multilocus genotypes of *P. psidii* sampled from different hosts in South America indicate that host species strongly influences rust population structure, as previously reported for *P. psidii* populations from Brazil (Graca et al., 2011). Distinct MGs are uniquely associated with specific hosts in Brazil, Paraguay, and Uruguay. All five *Eucalyptus* spp. isolates from Uruguay (UR) had the same unique MG, identical to one of the seven MGs sampled on *Eucalyptus* spp. from Brazil (EU). Similarly, the *P. guajava* isolate collected in Paraguay (PY) possessed an MG that is identical to that found in the *P. guajava*-derived population from Brazil (PG). Although the number of samples from PY and UR are reduced, the detection of identical MGs in UR and EU groups, and also in PG and PY, indicates that *P. psidii* population are not structured according to geographic location in South America. This finding suggests that urediniospores can perhaps move freely among the Brazil, Uruguay, and Paraguay, possibly by dispersal in the airstream. Further studies with a more representative number of isolates, collected on different hosts across several South American countries are needed to confirm this widespread dispersal of *P. psidii* urediniospores.

In contrast with the South American groups, all 50 rust isolates collected on nine different hosts in the Hawaiian Islands (Maui, Oahu, Kauai, and Hawaii/"Big Island") share a single, unique MG, indicating a recent introduction of a single rust genotype across the four Hawaiian Islands, as previously hypothesized (Uchida et al., 2006; Loope, 2010). The presence of a single MG across different host species indicates lack of selection by host

species on the rust population in Hawaii. Because the unique rust MG found in Hawaii does not infect *P. guajava* plants (Loope, 2010), concerns continue about the risks associated with the introduction of a new rust genotype capable to infect this host and other Myrtaceae species. The same unique MG detected across all Hawaiian isolates was also detected in California, suggesting that California is a potential source of the *P. psidii* introduction into the Hawaiian Islands. The hypothesis that the trade of Myrtaceae plants between California and Hawaii resulted in the introduction of *P. psidii* into the Hawaiian Islands, has been widely proposed (Uchida et al., 2006; Loope, 2010). The fact that the MG that occurs across four Hawaiian Islands on nine distinct hosts is also identical to the MG found in California, supports the hypothesis that the Hawaiian rust MG came from California by trade of ornamental plant species. However, the evolutionary origin of the MG in California and Hawaii remains unknown. Additional rust isolates from California, as well as isolates from Florida, Mexico, Central America, and the Caribbean Islands are going to be analyzed to address this question. Population genetic studies on cereal rusts demonstrated that movement of infected plant material was responsible for the spread of different rust species between continents (Wellings and McIntosh, 1990; HovmØller et al., 2008; Ordonez et al., 2010). The MG comprising all isolates from Hawaii and California is distinct from all currently known MGs from South America. Thus, it appears that the Hawaiian and Californian isolates did not come directly from Brazil, Paraguay or Uruguay. However, it should be noted that the Hawaiian genotype of *P. psidii* had several alleles in common with South American genotypes, indicating some relative similarities between the Hawaiian and Californian isolates with the Brazilian ones.

Selection by host species in South America was also observed on the PCA plot (Figure 2). The first two axes explained 82% of the total observed difference among rust groups. The lack of selection by host species among the 50 rust isolates collected on nine Myrtaceae species in Hawaii, was also detected on the PCA plot. The same pattern was observed on a NJ-tree, two isolates from California had identical MGs to the Hawaiian isolates, and were clustered together, as expected (Figure 3). A MJ-network was constructed to allow inferences about the relationship among haplotypes of the 11 rust groups. Strong differentiation by host species among the South American groups was also observed on the MJ-network (Figure 4). The two haplotypes on HI and CA groups were the most distinct haplotypes on the entire network, preceded by several median vectors (or missing haplotypes). No South American haplotype was found that is identical to those found in Hawaii and California, indicating that the rust genotype introduced into Hawaii did not come directly from South America. Based on

the NJ-dendrogram, the MC was the South American group more closely related to the HI and CA group.

It is perhaps possible to determine the origin of the *P. psidii* genotype that was introduced into California and Hawaii. To examine pathways of pathogen dispersal, rust isolates should be collected on different host species in South America, Central America, Mexico, in some Caribbean Islands, Florida, and other locations in California. The excess of median vectors on the MJ-network branches near to the HI and CA indicates that the haplotypes are missing that gave rise to the HI and CA haplotypes. Isolates from Florida, Central America and Caribbean Islands could perhaps provide information to help fill in the missing haplotypes.

The high levels of H_O (Birky, 1996; De Meeus and Balloux, 2004; Halkett et al., 2005), generating strong negative values of F_{IS}, on each rust group indicates high rate of clonal reproduction across all rust groups. Evidence of clonal reproduction was also supported by high values of I_A and r_D, indicating significant linkage disequilibrium. Similarly, no evidence of sexual reproduction was reported in Brazilian *P. psidii* groups (Graça et al., 2011). Due to the absence of sexual reproduction, it is believed that the low level of variation observed among isolates from *Eucalyptus* is generated by rare mutation events or some sort of recombination.

Hawaiian isolates all belong to the same genotype, regardless of their host species or geographic location. Thus, the introduction of any new *P. psidii ge*notypes into Hawaii represents a potential invasive pathogen risk for Myrtaceous trees. Furthermore, potential recombination of among genotypes could create new genotypes with unknown risks. For these reasons, it seems prudent to avoid the introductions of new *P. psidii* genotypes into Hawaii and elsewhere, and studies should continue to better assess invasive risks posed by diverse genotypes.

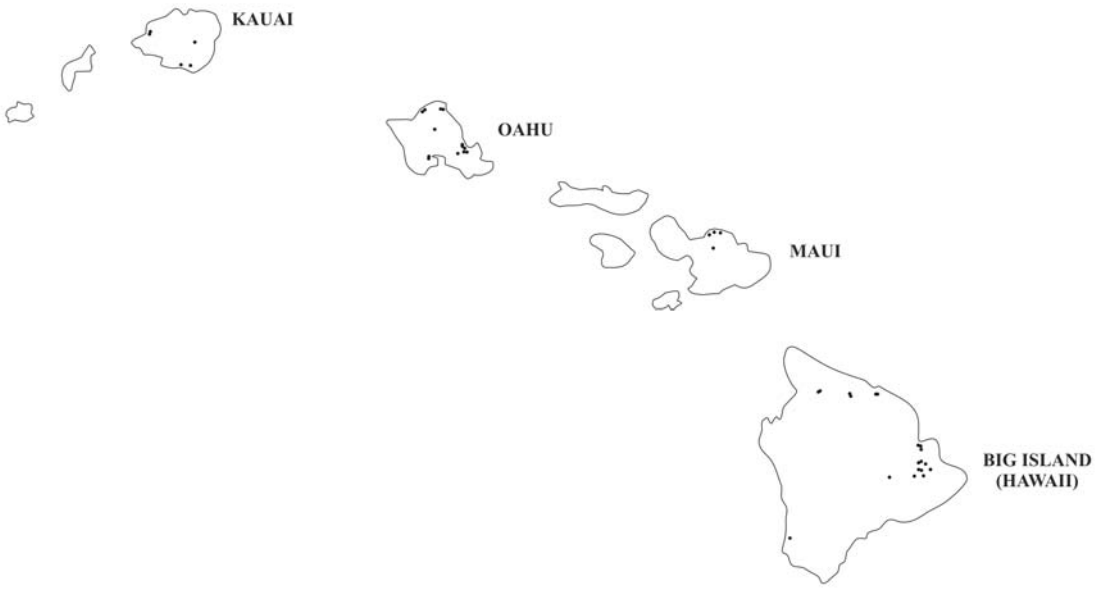

Figure 1. Map of the *Puccinia psidii* sample locations in Hawaii. Black dot represents the location of each isolate based on GPS coordinates.

Table 1. Sources of newly collected *Puccinia psidii* isolates from Myrtaceae species in Brazil, Paraguay, Uruguay, California, and Hawaii, analyzed in addition to the 148 rust isolates from Graca et al., 2011.

Country	Host	State	No. of isolates
Brazil	*Psidium araca*	Espirito Santo	1
	Eucalyptus spp.	Alagoas	1
		Mato Grosso	12
	Syzigium jambos	Mato Grosso	1
Paraguay	*P. guajava*	Asuncion	2
Uruguay	*Eucalyptus spp.*	Cerro Colorado	5
USA	*Myrtle communis*	California/Fallbro	1
	Melaleuca	California/San	1
	Metrosideros	Hawaii/Oahu	3
		Hawaii/Kauai	2
		Hawaii/Big Island	4
	M. excels	Hawaii/Oahu	1
	Eugenia koolauensis	Hawaii/Oahu	3
	Rodomyrthus	Hawaii/Kauai	2
	Myrtle communis	Hawaii/Maui	1
	S. samarangense	Hawaii/Big Island	1
	M. quinquinervia	Hawaii/Oahu	1
		Hawaii/Kauai	1
		Hawaii/Big Island	2
	S. cumini	Hawaii/Oahu	1
	S. jambos	Hawaii/Oahu	10
		Hawaii/Kauai	2
		Hawaii/Maui	2
		Hawaii/Big Island	14
Brazil (Graca et al., 2011)			148
Total			221

Table 2. Primer sequences and characteristics of eight microsatellite markers (adapted from Zhong et al., 2008) used on to characterize *Puccinia psidii* populations from Brazil, Paraguay, Uruguay, and the US states: California and Hawaii.

Locus	GenBank accession no.	Primer sequence (5' - 3')	gc%	Repeat motif	No. of alleles	Size range
PpSSR012	EF523501	F: TTCAATCCCCATAAGGCTTTC R: AAATCCTGAGTCTTCTTCCCC	42.86 47.62	$(AG)_9$	5	229 - 239
PpSSR014	EF523502	F: TTCGACATCCAACGCTCTCAT R: AAAGGCTAAGTGAATGGGCA	47.62 45	$(AG)_{13}$	4	206-214
PpSSR018	EF523503	F: AGCCTTCTCTCTCCTCCGTTA R: TCAGGAAGGACAAGACCAAGT	52.38 47.62	$(AG)_9$	4	162-172
*PpSSR022**	EF523504	F: CCTTTAGGCTGTGGTTTCCA R: GCCCACTCTGTCAAGAGGAA	50 55	$(AG)_{12}$	6	147-159
*PpSSR087**	EF523507	F: AAGAACGTGAACGGGAATGA R: GAAATGCCAGACGAAGGGTA	45 50	$(AG)_{14} + G_6 + A_9$	8	154-178
*PpSSR102**	EF523508	F: TGACTTTAATCATCTTCAAAACCAA R: ACCAATCCCCTTCCTTCATC	28 50	$T_7 + (AG)_{22} + T_7$	4	140-178
*PpSSR146**	EF523510	F: TTGGTAAAGAGGAGGGGATTC R: TCAGCACCAACCATTACCTTC	47.62 47.62	$(AG)_{73}$	7	205-249
*PpSSR195**	EF523513	F: GAACGAACCCAAACTTTCCA R: GGAAAGGAATGAGATTGAACACA	45 39.13	$(AG)_{18}$	4	208-226

***** New set of primers and its sequences designed on the software Primer3 based on the *P. psidii* microsatellite sequences deposited in the GenBank (Zhong et al., 2008).

Table 3. Overall measures of single-locus statistics of *Puccinia psidii* subpopulations from seven hosts (EU, SJ, PG, PA, SC, MC, and EG) in Brazil, from *Eucalyptus grandis* in Uruguay (UR), from *Psidium guajava* in Paraguay (PY) and from nine Myrtaceae species (*Metrosideros polymorpha, M. excelsa, Eugenia koolauensis, Rodomyrthus tomentosa, Myrtle communis, Syzigium samarangense, M. quinquinervia, S. cumini,* and *S. jambos*) in Hawaii (HI) and from *Melaleuca leucodendron* and *M. communis* collected in California (CA).

Parameters[a]	Host[b]										
	EU	**SJ**	**PG**	**PA**	**SC**	**MC**	**EG**	**UR**	**PY**	**HI**	**CA**
No. of alleles	2.000 (0.267)	1.625 (0.183)	1.375 (0.183)	1.500 (0.189)	1.625 (0.183)	1.875 (0.125)	1.250 (0.164)	1.625 (0.183)	1.375 (0..183)	1.875 (0..125)	1.875 (0..125)
No. of effective alleles	1.718 (0.172)	1.625 (0.183)	1.375 (0.183)	1.500 (0.189)	1.625 (0.183)	1.875 (0.125)	1.250 (0.164)	1.625 (0.183)	1.375 (0.183)	1.875 (0.125)	1.875 (0.125)
Shannon index *I*	0.529 (0.120)	0.433 (0.127)	0.260 (0.127)	0.347 (0.131)	0.433 (0.127)	0.607 (0.087)	0.173 (0.113)	0.433 (0.127)	0.260 (0.127)	0.607 (0.087)	0.607 (0.087)
H_O	0.660 (0.160)	0.625 (0.183)	0.375 (0.183)	0.500 (0.189)	0.625 (0.183)	0.875 (0.125)	0.250 (0.164)	0.625 (0.183)	0.375 (0.183)	0.875 (0.125)	0.875 (0.125)
H_E	0.361 (0.082)	0.313 (0.091)	0.188 (0.091)	0.250 (0.094)	0.313 (0.091)	0.438 (0.063)	0.125 (0.082)	0.313 (0.091)	0.188 (0.091)	0.438 (0.063)	0.438 (0.063)
F_{IS}	-0.794 (0.097)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)	-1.000 (0.000)
No. of Haplotypes	8	2	2	2	2	2	2	2	2	2	2
*PVA**	1	1	0	1	2	5	3	1	0	4	

[a] H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = fixation index; and *PVA* = number of private alleles.
[b] Numbers in parentheses = standard error.
* Isolates from Hawaii and California had identical multilocus genotypes (MG), so the number of *PVA* was calculated together.

Table 4. Analysis of molecular variance (AMOVA), based on R_{ST} statistics, among *Puccinia psidii* isolates collected on different hosts in Brazil, Uruguay, Paraguay, and in USA (Hawaii and California).

Source	**d.f.**	**Sum of squares**	**Percentage of variation**	***P***	**Fixation index**
Among rust groups	10	58513.05	93.62	0.001	0.936
Among individuals within groups	210	77.19	-6.20	1.000	-0.970
Within individuals	221	5424.50	12.58	0.001	0.874
Total	441	64014.75	100		

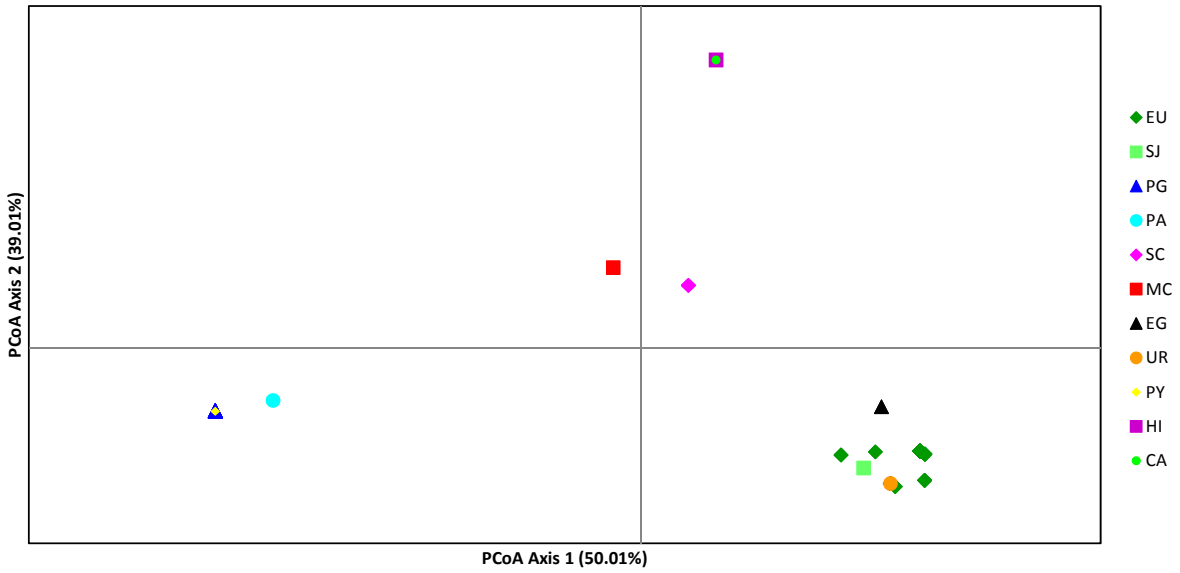

Figure 2. Principal coordinate analysis (PCA) plot of microsatellite multilocus genotypes (MGs) of 221 *Puccinia psidii* isolates collected from seven hosts in Brazil (EU, SJ, PG, PA, SC, MC, and EG), *Eucalyptus grandis* in Uruguay (UR), *Psidium guajava* in Paraguay (PY), and nine Myrtaceae species (*Metrosideros polymorpha, M. excelsa, Eugenia koolauensis, Rodomyrthus tomentosa, Myrtle communis, Syzigium samarangense, M. quinquinervia, S. cumini,* and *S. jambos*) in Hawaii (HI) and two Myrtaceae species (*Melaleuca leucodendron* and *M. communis*) in California (CA). The first two axes explain 89.02% of the observed variation.

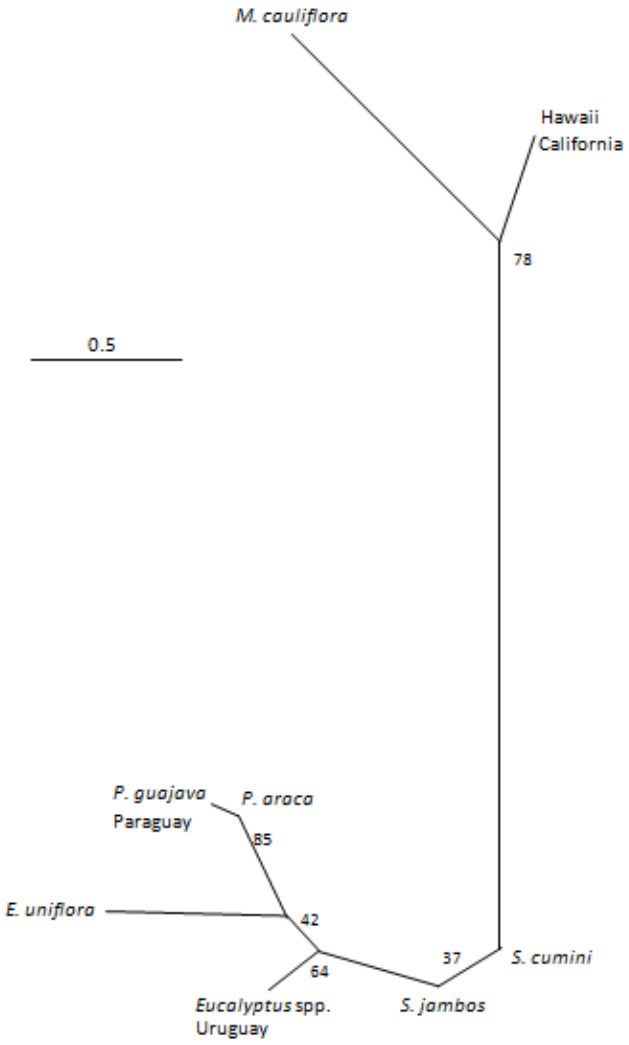

Figure 3. Neighbor joining tree of the microsatellite multilocus genotypes showing the relationships among *Puccinia psidii* isolates from seven hosts in Brazil (EU, SJ, PG, PA, SC, MC, and EG), *Eucalyptus grandis* in Uruguay (UR), *Psidium guajava* in Paraguay (PY), and nine Myrtaceae species (*Metrosideros polymorpha, M. excelsa, Eugenia koolauensis, Rodomyrthus tomentosa, Myrtle communis, Syzigium samarangense, M. quinquinervia, S. cumini,* and *S. jambos*) in Hawaii (HI) and two Myrtaceae species (*Melaleuca leucodendron* and *M. communis*) in California (CA). Bootstrap values based on 1000 replications are shown. Branch length corresponds to genetic distance between groups.

Table 5. *Puccinia psidii* pairwise population R_{ST} estimated between isolate hosts of origin. R_{ST} values below diagonal. Probability values based on 999 permutations are shown above diagonal.

Host	Host of origin										
	EU	**SJ**	**PG**	**PA**	**SC**	**MC**	**EG**	**UR**	**PY**	**HI**	**CA**
EU	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.349	0.001	0.001	0.001
SJ	0.767	0.000	0.001	0.161	0.137	0.001	0.050	0.006	0.186	0.001	0.003
PG	0.787	0.559	0.000	0.208	0.001	0.001	0.001	0.001	0.389	0.001	0.001
PA	0.798	0.154*	0.099*	0.000	0.051	0.002	0.003	0.001	0.422	0.001	0.003
SC	0.659	0.149*	0.612	0.323	0.000	0.001	0.003	0.003	0.099	0.001	0.001
MC	0.974	0.695	0.970	0.933	0.863	0.000	0.001	0.001	0.005	0.001	0.002
EG	0.658	0.477*	0.844	0.889	0.570	0.965	0.000	0.001	0.018	0.001	0.015
UR	0.000*	0.459	0.829	0.820	0.462	0.965	0.719	0.000	0.001	0.001	0.001
PY	0.757	0.167*	0.000*	0.089*	0.280*	0.936	0.893	0.787	0.000	0.001	0.014
HI	0.966	0.891	0.955	0.920	0.922	0.881	0.945	0.944	0.923	0.000	0.426
CA	0.981	0.782	0.982	0.954	0.901	0.904	0.969	0.970	0.952	0.000*	0.000

* All values significant at $P < 0.05$, except those indicated with *, where $P > 0.05$.

Table 6. Likelihood estimates of Weir & Cokerhan's Theta (θ) between all pairs of *Puccinia psidii* populations from seven hosts in Brazil. Theta θ values below diagonal. Probability values based on 999 permutations are shown above diagonal.

Host	Host of origin										
	EU	**SJ**	**PG**	**PA**	**SC**	**MC**	**EG**	**UR**	**PY**	**HI**	**CA**
EU	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.646	0.001	0.001	0.001
SJ	0.062*	0.000	0.001	0.032	0.035	0.032	0.066	0.011	0.077	0.001	0.079
PG	0.566	0.646	0.000	0.001	0.001	0.001	0.001	0.001	1.000	0.001	0.001
PA	0.466*	0.469*	0.069*	0.000	0.028	0.103	0.120	0.015	0.106	0.001	0.126
SC	0.452*	0.481*	0.681	0.517	0.000	0.031	0.061	0.001	0.071	0.001	0.054
MC	0.542	0.506	0.704	0.492	0.506	0.000	0.094	0.017	0.099	0.001	0.103
EG	0.382*	0.392	0.751	0.671	0.596	0.595	0.000	0.049	0.316	0.002	0.331
UR	-0.049	-0.016*	0.645	0.456	0.433*	0.489*	0.369*	0.000	0.053	0.001	0.049
PY	0.478*	0.491	-0.148	-0.107	0.536	0.494	0.722	0.471	0.000	0.001	0.328
HI	0.466*	0.442*	0.591	0.457*	0.418*	0.470*	0.504	0.428*	0.465*	0.000	1.000
CA	0.447*	0.409	0.675	0.442	0.383	0.372	0.520	0.387	0.444	-0.149	0.000

*$\theta < 0.5$ indicate significantly low differentiation between populations ($P < 0.05$).

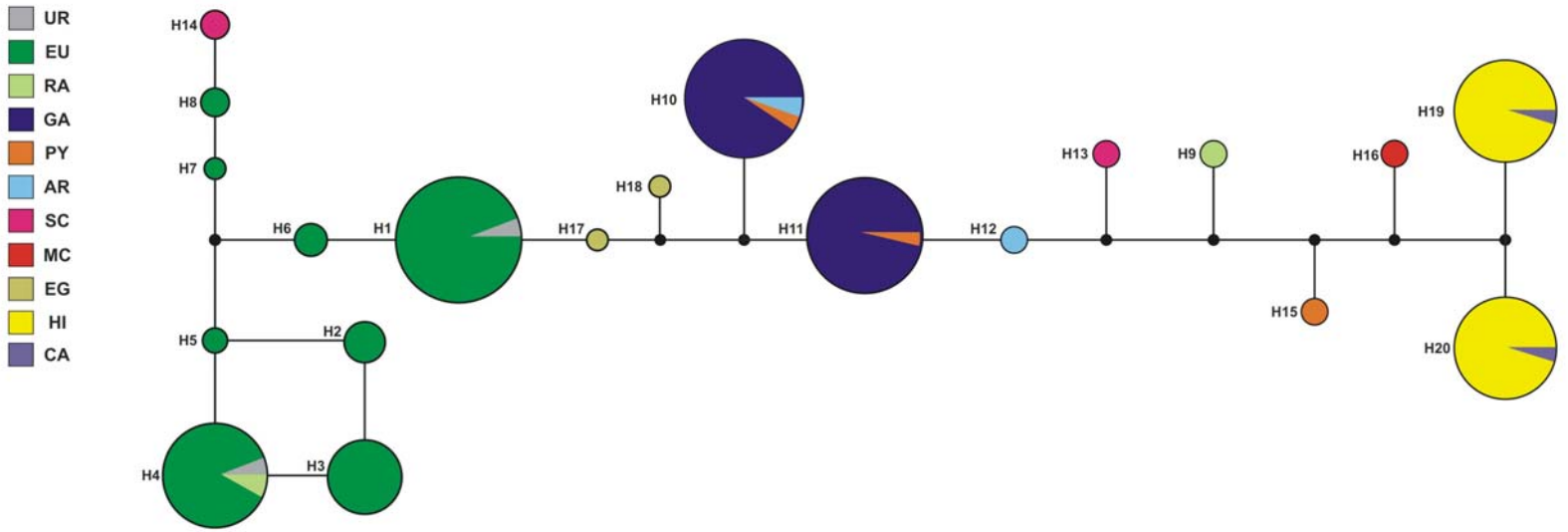

Figure 4. Median-joining (MJ) network of *Puccinia psidii* collected on seven hosts in Brazil (EU, SJ, PG, PA, SC, MC, and EG), from *Eucalyptus grandis* in Uruguay (UR), from *Psidium guajava* in Paraguay (PY), from nine Myrtaceae species in Hawaii (HI) (*Metrosideros polymorpha, M. excelsa, Eugenia koolauensis, Rodomyrthus tomentosa, Myrtle communis, Syzigium samarangense, M. quinquinervia, S. cumini,* and *S. jambos*), and from *Melaleuca leucodendron* and *M communis* collected in California (CA). Sizes of haplotypes circles indicate haplotypes frequencies. Haplotypes from different host populations are color coded.

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GENERAL CONCLUSIONS

The present work allows us to conclude that:

1) A new race of *Puccinia psidii* (race 4) was able to overcome the resistant gene *Ppr-1* breakdown on *Eucalyptus grandis*, this new race was also capable to infect a large number of clones resistant to race 1 predominant;

2) Indicative of selection by host and high rate of clonal reproduction in *P. psidii* population from Brazil were revealed by microsatellite multilocus genotypes (MG). Indicating that host species affect the genetic structure of *P. psidii* in Brazil;

3) No evidences of selection by host species was observed in Hawaii and California. All rust isolates collected on nine myrtaceous hosts in Hawaiian Islands presented a unique MG. The same unique MG observed in Hawaii, was also detected on isolates from two different hosts in California, suggesting that California might be the source of *P. psidii* introduction into Hawaii. However, the evolutionary origin of the MG in California and Hawaii remains unknown. Additional rust isolates from California, as well as isolates from Florida, Mexico, Central America, and the Caribbean Islands are going to be analyzed to address this question.The MG comprising all isolates from Hawaii and California is distinct from the MGs found in South America so far, suggesting that the Hawaiian and Californian isolates did not come directly from South America.

4. APPENDIX

Table 1S. Geographical coordinates of each *Puccinia psidii* isoltate used on this study.

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
pop = EU						
SUZ5_EU	*E. urophylla x E. grandis*	EU1	Brazil/BA	S17.9849	W39.9763	105
SUZ6_EU	*E. urophylla x E. grandis*	EU2	Brazil/BA	S18.3350	W39.7034	21
SUZ7_EU	*E. urophylla x E. grandis*	EU3	Brazil/BA	S18.3359	W39.7015	27
SUZ8_EU	*E. urophylla X E. grandis*	EU4	Brazil/BA	S18.4865	W39.9849	78
SUZ9_EU	*E. urophylla x E. grandis*	EU5	Brazil/BA	S16.1744	W39.6153	268
SUZ10_EU	*E. urophylla x E. grandis*	EU6	Brazil/BA	S16.1774	W39.6320	301
SUZ11_EU	*E. grandis (semente)*	EU7	Brazil/SP	S16.1721	W39.6489	356
SUZ12_EU	*E. grandis (semente)*	EU8	Brazil/SP	S16.0375	W39.1401	79
SUZ13_EU	*E. urophylla X E. grandis*	EU9	Brazil/BA	S16.1132	W39.3158	113
SP1_EU	*E. grandis (7)*	EU10	Brazil/SP	S23.8681	W47.9055	715
SP2_EU	*E. grandis (7)*	EU11	Brazil/SP	S23.8679	W47.9044	713
SP3_EU	*E. grandis (7)*	EU12	Brazil/SP	S23.8681	W47.9049	710
SP4_EU	*E.grandis (clone 24)*	EU13	Brazil/SP	S23.8997	W47.8332	755
SP6_EU	*E. grandisXE.dunni (clone 581)*	EU14	Brazil/SP	S23.9117	W47.6076	960
SP7_EU	*E. grandis (clone 617)*	EU15	Brazil/SP	S23.9117	W47.6076	960
SP11_EU	*E. grandis (semente)*	EU16	Brazil/SP	S24.1466	W49.2206	860
SP12_EU	*E. grandis (semente)*	EU17	Brazil/SP	S24.1464	W49.2210	865
SP13_EU	*E. grandis (semente)*	EU18	Brazil/SP	S24.1467	W49.2162	850
SP14_EU	*E. grandis (clone 24)*	EU19	Brazil/SP	S24.1468	W49.2073	850
SP15_EU	*E. grandis (clone 24)*	EU20	Brazil/SP	S24.1470	W49.2100	850
SP16_EU	*E. grandis (semente)*	EU21	Brazil/SP	S24.1595	W49.2059	880
SP17_EU	*E. grandis (semente)*	EU22	Brazil/SP	S23.6145	W48.0831	687
SP19_EU	*E. grandis (24)*	EU23	Brazil/SP	S23.2627	W48.4249	662
SP21_EU	*E. grandis (clone 7)*	EU24	Brazil/SP	S23.2626	W48.4248	655

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
SP22_EU	*E. grandis (semente)*	EU25	Brazil/SP	S23.2954	W48.4462	650
SP25_EU	*E. grandis (m-33)*	EU26	Brazil/SP	S23.6434	W46.0089	780
SP26_EU	*E. grandis (m-33)*	EU27	Brazil/SP	S23.6401	W46.0087	790
SP27_EU	*E. grandis (m-25)*	EU28	Brazil/SP	S23.6480	W45.9895	930
RJ4_EU	*Eucalyptus spp.*	EU29	Brazil/RJ	S21.7615	W41.2882	12
RJ15_EU	*Eucalyptus spp.*	EU30	Brazil/ES	S21.0550	W41.3621	103
RJ18_EU	*Eucalyptus spp.*	EU31	Brazil/ES	S20.7570	W41.5749	629
RJ19_EU	*Eucalyptus spp.*	EU32	Brazil/ES	S20.7966	W41.7531	615
IS1_EU	*E. dunnii (semente)*	EU33	Brazil/SC	S27.4523	W50.0895	880
IS2_EU	*E. dunnii (clone 13)*	EU34	Brazil/SC	S27.4494	W50.0941	878
IS3_EU	*E. grandis (clone 360)*	EU35	Brazil/SC	S27.4493	W50.0933	869
IS4_EU	*E. urograndis X viminalis*	EU36	Brazil/SC	S27.4491	W50.0928	876
IS5_EU	*E. urograndis X grandisglobulus*	EU37	Brazil/SC	S27.4489	W50.0939	878
IS6_EU	*E. dunnii*	EU38	Brazil/SC	S27.4338	W50.0319	878
IS7_EU	*E. dunnii (1007)*	EU39	Brazil/SC	S27.4236	W50.0319	867
IS9_EU	*Eucalyptus spp. (clone 4013)*	EU40	Brasil/PR	S24.2184	W50.8127	809
IS10_EU	*Eucalyptus spp. (clone 4013)*	EU41	Brasil/PR	S24.2242	W50.8023	781
IS12_EU	*E. grandis (semente)*	EU42	Brasil/PR	S24.1706	W50.5069	909
ES6_EU	*E. grandis (3918)*	EU43	Brasil/ES	S18.5843	W39.8928	51
ES7_EU	*E. grandis (3918)*	EU44	Brasil/ES	S18.4412	W39.7557	29
ES8_EU	*E. grandis (3918)*	EU45	Brasil/ES	S19.8223	W40.2321	62
ES9_EU	*E. grandis (3918)*	EU46	Brasil/ES	S19.8292	W40.2400	72
ES10_EU	*E. grandis (3918)*	EU47	Brasil/ES	S19.8308	W40.2533	69
ES11_EU	*E. grandis (3918)*	EU48	Brasil/ES	S19.7852	W40.2071	52
ES12_EU	*E. grandis (3918)*	EU49	Brasil/ES	S19.8196	W40.1540	39
ES13_EU	*E. grandis (3918)*	EU50	Brasil/ES	S19.6611	W40.1896	63
ES14_EU	*E. grandis (3918)*	EU51	Brasil/ES	S19.8399	W40.1028	26

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
ES15_EU	*E. grandis (3918)*	EU52	Brasil/ES	S19.8824	W40.1187	38
ES16_EU	*E. grandis (3918)*	EU53	Brasil/ES	S19.9131	W40.1335	31
ES17_EU	*E. grandis (3918)*	EU54	Brasil/ES	S19.8653	W40.2653	66
ES23_EU	*Eucalyptus spp.*	EU55	Brasil/ES	S20.2413	W41.3369	1036
V2_EU	*E. urophylla x E. grandis (clone 874)*	EU56	Brazil/BA	S15.6196	W39.2675	124
V3_EU	*E. urophylla x E. grandis (clone 1006)*	EU57	Brazil/BA	S15.6196	W39.2675	124
V4_EU	*E. urophylla x E. grandis (clone 1004)*	EU58	Brazil/BA	S15.6195	W39.2675	124
V5_EU	*E. urophylla x E. grandis (clone 865)*	EU59	Brazil/BA	S15.6196	W39.2675	124
V6_EU	*E. urophylla x E. grandis (clone 978)*	EU60	Brazil/BA	S15.6197	W39.2675	124
V8_EU	*E. urophylla x E. grandis (clone 1004)*	EU61	Brazil/BA	S16.1185	W39.2119	89
V9_EU	*E. urophylla x E. grandis (874)*	EU62	Brazil/BA	S16.1185	W39.2119	89
V10_EU	*E. urophylla x E. grandis (6021)*	EU63	Brazil/BA	S16.1185	W39.2119	89
V11_EU	*E. urophylla x E. grandis (865)*	EU64	Brazil/BA	S16.3488	W39.5986	192
V13_EU	*E. urophylla x E. grandis (1004)*	EU65	Brazil/BA	S16.3488	W39.5850	185
OP24_EU	*Eucalyptus* spp.	EU66	Brazil/MG	S20.6685	W43.1087	622
UENF7_EU	*E. grandis*	EU67	Brazil/MG	S21.0747	W42.4998	632
UFV2_EU	*E. grandis*	EU68	Brazil/SP	S22.5986	W48.8003	550
EUBA1_EU	E. urophylla x E. grandis (6021)	EU69	Brazil/BA	S18.4865	W39.9849	78
MS1_EU	*Eucalyptus* spp.	EU70	Brazil/MS	S20.9973	W51.7766	682
MS2_EU	*Eucalyptus* spp.	EU71	Brazil/MS	S20.9949	W51.7739	677
MS3_EU	*Eucalyptus* spp.	EU72	Brazil/MS	S20.9837	W51.7771	682
MS4_EU	*Eucalyptus* spp.	EU73	Brazil/MS	S20.9832	W51.7772	690
MS5_EU	*Eucalyptus* spp.	EU74	Brazil/MS	S20.9832	W51.7772	690
MS6_EU	*Eucalyptus* spp.	EU75	Brazil/MS	S20.9837	W51.7771	682
MS7_EU	*Eucalyptus* spp.	EU76	Brazil/MS	S20.9832	W51.7772	690
MS8_EU	*Eucalyptus* spp.	EU77	Brazil/MS	S20.9792	W51.7255	690
MS9_EU	*Eucalyptus* spp.	EU78	Brazil/MS	S20.9747	W51.7866	689

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
MS10_EU	*Eucalyptus* spp.	EU79	Brazil/MS	S20.9629	W51.8124	689
MS11_EU	*Eucalyptus* spp.	EU80	Brazil/MS	S20.9629	W51.8124	689
MS12EU	*Eucalyptus* spp.	EU81	Brazil/MS	S21.0170	W51.7765	693
MS13EU	*Eucalyptus* spp.	EU82	Brazil/MS	S20.9883	W51.8181	684
AL01_EU	*Eucalyptus* spp.	EU83	Brazil/AL	?	?	?
pop = SJ						
RJ6A_RA	*Syzigium jambos*	SJ1	Brazil/RJ	S21.7486	W41.3087	9
SUZ14_RA	*S. jambos*	SJ2	Brazil/MG	S20.1238	W42.4616	316
CB1_RA	*S. jambos*	SJ3	Brazil/MG	S19.2971	W42.3904	233
MS15_RA	*S. jambos*	SJ4	Brazil/MS	S20.8933	W51.7758	335
pop = PG						
SUZ1_GA	*Psidium guajava*	PG1	Brazil/MG	S20.1251	W42.4622	308
SUZ2_GA	*P. guajava*	PG2	Brazil/MG	S20.0565	W42.4335	288
SUZ15_GA	*P. guajava*	PG3	Brazil/MG	S20.4993	W42.8766	507
SP18_GA	*P. guajava*	PG4	Brazil/SP	S23.2627	W48.4249	660
SP24_GA	*P. guajava*	PG5	Brazil/SP	S23.6434	W46.0094	750
SP28_GA	*P. guajava*	PG6	Brazil/SP	S23.6149	W46.0088	780
SP29_GA	*P. guajava*	PG7	Brazil/SP	S23.6145	W46.0087	790
RJ1_GA	*P. guajava*	PG8	Brazil/MG	S22.1508	W43.1587	274
RJ2_GA	*P. guajava*	PG9	Brazil/RJ	S22.4070	W43.0480	1306
RJ3_GA	*P. guajava*	PG10	Brazil/RJ	S22.3402	W43.8528	821
RJ10_GA	*P. guajava*	PG11	Brazil/RJ	S21.7661	W41.2712	18
RJ12_GA	*P. guajava*	PG12	Brazil/RJ	S21.2617	W41.3266	28
RJ13_GA	*P. guajava*	PG13	Brazil/ES	S21.0576	W41.3598	76
RJ14_GA	*P. guajava*	PG14	Brazil/ES	S21.0572	W41.3600	80
RJ16_GA	*P. guajava*	PG15	Brazil/ES	S20.9437	W41.3353	236
ES3_GA	*P. guajava*	PG16	Brazil/ES	S20.7515	W41.4886	127

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
ES4_GA	*P. guajava*	PG17	Brazil/ES	S21.0078	W40.8070	6
ES18_GA	*P. guajava*	PG18	Brazil/ES	S19.9540	W40.1499	5
ES21_GA	*P. guajava*	PG19	Brazil/ES	S20.3351	W41.1266	734
ES24_GA	*P. guajava*	PG20	Brazil/ES	S20.2364	W41.5213	775
OP23_GA	*P. guajava*	PG21	Brazil/MG	S20.6958	W43.2495	608
OP25_GA	*P. guajava*	PG22	Brazil/MG	S20.7081	W43.0364	777
UENF5_GA	*P. guajava*	PG23	Brazil/RJ	S21.3546	W41.0924	32
UENF6_GA	*P. guajava*	PG24	Brazil/RJ	S21.3546	W41.0924	32
PC1_GA	*P. guajava*	PG25	Brazil/MG	S20.8367	W42.9966	705
PC2_GA	*P. guajava*	PG26	Brazil/MG	S20.8364	W42.9965	698
PC3_GA	*P. guajava*	PG27	Brazil/MG	S20.8367	W42.9961	698
3PONTAS_GA	*P. guajava*	PG28	Brazil/MG	S21.3708	W45.4767	914
SP30_GA	*P. guajava*	PG29	Brazil/SP	S23.6145	W48.0831	687
RJ5_GA	*P. guajava*	PG30	Brazil/RJ	S21.8025	W41.2940	8
RJ9_GA	*P. guajava*	PG31	Brazil/RJ	S21.6900	W41.0555	7
RJ17_GA	*P. guajava*	PG32	Brazil/ES	S20.7900	W41.3889	141
IS11_GA	*P. guajava*	PG33	Brazil/PR	S24.2263	W50.5401	779
ES20_GA	*P. guajava*	PG34	Brazil/ES	S20.0378	W40.1834	8
ES22_GA	*P. guajava*	PG35	Brazil/ES	S20.2938	W41.2275	989
ES25_GA	*P. guajava*	PG36	Brazil/MG	S20.2996	W42.4739	582
OP1_GA	*P. guajava*	PG37	Brazil/MG	S20.4097	W43.0507	587
OP2_GA	*P. guajava*	PG38	Brazil/MG	S20.4085	W43.1008	614
OP3_GA	*P. guajava*	PG39	Brazil/MG	S20.3740	W43.2540	769
OP4_GA	*P. guajava*	PG40	Brazil/MG	S20.3836	W43.3370	765
OP5_GA	*P. guajava*	PG41	Brazil/MG	S20.3916	W43.4798	1079
OP6_GA	*P. guajava*	PG42	Brazil/MG	S20.3754	W43.5323	1245
OP7_GA	*P. guajava*	PG43	Brazil/MG	S20.3797	W43.5216	1167

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
OP8_GA	*P. guajava*	PG44	Brazil/MG	S20.4471	W43.5575	1272
OP9_GA	*P. guajava*	PG45	Brazil/MG	S20.4771	W43.5200	1387
OP10_GA	*P. guajava*	PG46	Brazil/MG	S20.3767	W43.5917	1193
OP11_GA	*P. guajava*	PG47	Brazil/MG	S20.3628	W43.6517	1181
OP12_GA	*P. guajava*	PG48	Brazil/MG	S20.4861	W43.5986	1033
OP13_GA	*P. guajava*	PG49	Brazil/MG	S20.5202	W43.6934	1021
OP14_GA	*P. guajava*	PG50	Brazil/MG	S20.5471	W43.7089	1073
OP15_GA	*P. guajava*	PG51	Brazil/MG	S20.5780	W43.7262	1054
OP16_GA	*P. guajava*	PG52	Brazil/MG	S20.6533	W43.7580	972
OP18_GA	*P. guajava*	PG53	Brazil/MG	S20.6463	W43.7263	963
OP19_GA	*P. guajava*	PG54	Brazil/MG	S20.6720	W43.6149	754
OP20_GA	*P. guajava*	PG55	Brazil/MG	S20.6877	W43.5025	753
OP21_GA	*P. guajava*	PG56	Brazil/MG	S20.7953	W43.4718	749
OP22_GA	*P. guajava*	PG57	Brazil/MG	S20.6841	W43.2252	807
UENF3_GA	*P. guajava*	PG58	Brazil/RJ	S21.7496	W41.3091	10
UENF4_GA	*P. guajava*	PG59	Brazil/RJ	S21.2971	W41.1626	24
RS2_GA	*P. guajava*	PG60	Brazil/RS	S30.5966	W51.5171	150
RS3_GA	*P. guajava*	PG61	Brazil/RS	S30.5959	W51.5014	150
RS6_GA	*P. guajava*	PG62	Brazil/RS	S30.6148	W51.4660	105
RS7_GA	*P. guajava*	PG63	Brazil/RS	S30.1503	W51.4801	110
pop = PA						
V1_AR	*Psidium araca*	PA1	Brazil/BA	S15.5903	W39.2892	120
OP17_AR	*P. araca*	PA2	Brazil/MG	S20.6463	W43.7263	963
SILV_AR						
pop = SC						
SP9_JAO	Syzigium cumini	SC1	Brazil/SP	S23.5512	W47.8978	555
SP23_JAO	S. cumini	SC2	Brazil/SP	S23.2953	W48.4461	652

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
UENF2_JAO	S. cumini	SC3	Brazil/RJ	S21.7483	W41.3088	8
RJ7_JAO	S. cumini	SC4	Brazil/RJ	S21.7288	W41.2640	10
pop = MC						
JBMG1_JBT	*Myrciaria cauliflora*	MC1	Brazil/MG	S20.6554	W42.8367	675
SP10_JBT	*M. cauliflora*	MC2	Brazil/SP	S23.5510	W47.8978	550
RJ11_JBT	*M. cauliflora*	MC3	Brazil/RJ	S21.7419	W41.2964	21
pop = EG						
PTMG1_PT	*Eugenia uniflora*	PT1	Brazil/MG	S20.7748	W42.8771	655
PITANGA_PT	*E. uniflora*	PT2	Brazil/MG	S20.7623	W45.8845	685
pop = UR						
URUGUAI_EU	*E. globulus*	UR1	Uruguai	S33.6307	W55.9576	600
Uruguai 2_ EU	*Eucalyptus*	UR2	Uruguai	S32.6381	W55.8257	135
UR-77_EU	*Eucalyptus*	UR3	Uruguai	S32.9015	W55.4126	150
UR-85_EU	*Eucalyptus*	UR4	Uruguai	S33.0683	W55.8409	130
UR-86b_EU	*Eucalyptus*	UR5	Uruguai	S31.6711	W55.9565	147
pop = PY						
PY01_GA	*P. guajava*	PY1	Paraguay	?	?	?
PY02_GA	*P. guajava*	PY2	Paraguay	?	?	?
pop = HI						
HW-1	*Syzigium jambos*	HI1	Hawaii/Oahu	N21.3439	W157.8081	305
HW-2	*E. koolauensis*	HI2	Hawaii/Oahu	N21.3756	W158.0309	100
HW-3	*S. jambos*	HI3	Hawaii/Oahu	N21.3754	W158.0259	75
HW-4	*S. jambos*	HI4	Hawaii/Oahu	N21.3754	W158.0259	75
HW-5	*S. jambos*	HI5	Hawaii/Oahu	N21.3754	W158.0259	75
HW-6	*S. jambos*	HI6	Hawaii/Oahu	N21.3754	W158.0259	75
HW-7	*Metrosideros excelsa*	HI7	Hawaii/Oahu	N21.3820	W157.8030	277

Population/Sample ID	Host	Code	Location	GPS coordinates		Elevation (m)
HW-8	*Metrosideros polymorpha*	HI8	Hawaii/Oahu	N21.3261	W157.7092	82
HW-9	*S. jambos*	HI9	Hawaii/Oahu	N21.3799	W157.8015	249
HW-10	*S. jambos*	HI10	Hawaii/Oahu	N21.3799	W157.8015	249
HW-11	*Rhodomyrtus tomentosa*	HI11	Hawaii/Kauai	N22.0831	W159.3814	191
HW-12	*S. jambos*	HI12	Hawaii/Kauai	N22.0421	W159.2411	645
HW-13	*S. jambos*	HI13	Hawaii/Kauai	N22.0736	W159.3947	197
501-HPPB	*S. jambos*	HI14	Hawaii/Hilo	N19.5438	W155.1257	525
501-HPPD	*S. jambos*	HI15	Hawaii/Hilo	N19.5105	W155.1347	668
501-HPPE	*Melaleuca quinquinervia*	HI16	Hawaii/Hilo	N19.5114	W155.1469	685
501-HPPF	*S. jambos*	HI17	Hawaii/Hilo	N19.5055	W155.1487	690
501-HPPG	*S. jambos*	HI18	Hawaii/Hilo	N19.5055	W155.1487	690
501-HPPL	*S. jambos*	HI19	Hawaii/Hilo	N19.6516	W155.0524	106
501-HPPH	*S. jambos*	HI20	Hawaii/Hilo	N19.5050	W155.1536	704
501-HPPI	*S. jambos*	HI21	Hawaii/Hilo	N19.5050	W155.1536	704
501-HPPJ	*S. jambos*	HI22	Hawaii/Hilo	N19.5834	W155.0769	282
501-HPPK	*S. jambos*	HI23	Hawaii/Hilo	N19.5834	W155.0769	282
501-HPPM	*S. jambos*	HI24	Hawaii/Hilo	N19.6516	W155.0524	106
501-RA5	*S. jambos*	HI25	Hawaii/Oahu	N21.3350	W157.8126	160
Oahu-1	*S. jambos*	HI26	Hawaii/Oahu	N21.4648	W157.9533	343
Oahu-2	*Metrosideros polymorpha*	HI27	Hawaii/Oahu	N21.4676	W157.9368	435
Oahu-3	*S. jambos*	HI28	Hawaii/Oahu	N21.4674	W157.9357	452
Oahu-4	*Melaleuca quinquinervia*	HI29	Hawaii/Oahu	N21.4671	W157.9429	424
Oahu-5	*M. polymorpha*	HI30	Hawaii/Oahu	N21.3331	W157.8020	147
Oahu-6	*Eugenia koolauensis*	HI31	Hawaii/Oahu	N21.6344	W158.0538	11
Oahu-7	*E. koolauensis*	HI32	Hawaii/Oahu	N21.6322	W158.0524	49

Oahu-8	*S. jambos*	HI33	Hawaii/Oahu	N21.6304	W158.0446	30
Population/Sample ID	**Host**	**Code**	**Location**	**GPS coordinates**		**Elevation (m)**
Oahu-10	*S. cumini*	HI34	Hawaii/Oahu	N21.6303	W158.0450	33
Hilo-11	*M. polymorpha*	HI35	Hawaii/Hilo	N19.6851	W155.0953	133
Hilo-12	*S. jambos*	HI36	Hawaii/Hilo	N19.6603	W155.0610	78
Hilo-13	*S. jambos*	HI37	Hawaii/Hilo	N19.5794	W155.1182	468
Hilo-14	*Syzygium samarangense*	HI38	Hawaii/Hilo	N19.6460	W155.0822	176
Hilo-15	*Metrosideros polymorpha*	HI39	Hawaii/Hilo	N19.7038	W155.0730	13
Hilo-16	*M. polymorpha*	HI40	Hawaii/Hilo	N19.7189	W155.1087	114
Hilo-17	*M. quinquinervia*	HI41	Hawaii/Hilo	N19.6488	W155.1217	315
Hilo-18	*S. jambos*	HI42	Hawaii/Hilo	N19.6488	W155.1217	315
Hilo-19	*M. polymorpha*	HI43	Hawaii/Hilo	N19.4964	W155.1456	681
PS-14	*Myrtus communis*	HI44	Hawaii/Maui	N20.7872	W156.3245	766
PS-16	*S. jambos*	HI45	Hawaii/Maui	N20.9071	W156.2717	253
PS-17	*S. jambos*	HI46	Hawaii/Maui	N20.8949	W156.2195	253
PS-47	*S. jambos*	HI47	Hawaii/Kauai	N22.1005	W159.6770	1049
PS-49	*R. tomentosa*	HI48	Hawaii/Kauai	N21.9230	W159.5011	252
PS-51	*M. polymorpha*	HI49	Hawaii/Kauai	N22.0728	W159.4003	215
PS-53	*M. polymorpha*	HI50	Hawaii/Kauai	N22.0658	W159.3957	181
pop = CA						
CA-1	*M. communis*	CA1	California/San Diego	?	?	?
CA-2	*Melaleuca lecondendron*	CA2	California/Fallbrook	?	?	?