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Rust disease of eucalypts, caused by *Puccinia psidii*, did not originate via host jump from guava in Brazil

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Abstract

The rust fungus, *Puccinia psidii*, is a devastating pathogen of introduced eucalypts (*Eucalyptus* spp.) in Brazil where it was first observed in 1912. This pathogen is hypothesized to be endemic to South and Central America and to have first infected eucalypts via a host jump from native guava (*Psidium guajava*). Ten microsatellite markers were used to genotype 148 *P. psidii* samples from eucalypts and guava plus five additional myrtaceous hosts across a wide geographic range of south-eastern Brazil and Uruguay. Principal coordinates analysis, a Bayesian clustering analysis and a minimum-spanning network revealed two major genetic clusters among the sampled isolates, one associated with guava and another associated with eucalypts and three additional hosts. Multilocus genotypes infecting guava differed by multiple mutational steps at eight loci compared with those infecting eucalypts. Approximate Bayesian computation revealed that evolutionary scenarios involving a coalescence event between guava- and eucalypt-associated pathogen populations within the past 1000 years are highly unlikely. None of the analyses supported the hypothesis that eucalypt-infecting *P. psidii* in Brazil originated via host jump from guava following the introduction of eucalypts to Brazil approximately 185 years ago. The existence of host-associated biotypes of *P. psidii* in Brazil indicates that this diversity must be considered when assessing the invasive threat posed by this pathogen to myrtaceous hosts worldwide.

Keywords: emerging disease, host jump, microsatellite, pathogen diversity, population structure

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Introduction

Novel associations of pathogens and hosts are of great scientific and economic interest and emerging diseases, such as Dutch elm disease, bat white nose syndrome, sudden oak death and amphibian decline have captured the focus of scientists and public alike (Brasier 2001; Rizzo *et al*. 2005; Fisher *et al*. 2009, 2012; Frick *et al*. 2010; Kupferschmidt 2012). Novel plant disease associations can result from host jumps or host shifts some of which may be associated with plant domestication (Couch *et al*. 2005; Stukenbrock *et al*. 2007; Stukenbrock & McDonald 2008). Novel associations can also result from pathogen hybridization or the introduction of a pathogen to a new location with previously unexposed or naïve hosts. Host shifts are generally defined as movement of a pathogen between two closely related hosts, while a host jump is defined as the movement of a pathogen between two hosts separated by larger taxonomic distances (Stukenbrock & McDonald 2008). Because differences between host shifts and host jumps are often ambiguous, hereafter the term 'host jump' will be used when referring to these events. The role of pathogen hybridization as an

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important evolutionary force in the establishment of novel diseases is increasingly recognized (Brasier 2001; Farrer *et al.* 2011; Goss *et al.* 2011), and it is possible that the significance of hybridization in disease emergence has been historically underestimated. A third mechanism for the development of novel disease associations results from the introduction of a pathogen to a new region, generally through anthropogenic means, where it is able to infect a previously unexposed or naïve host (Anderson *et al.* 2004). Classic examples of such introduced plant pathogens include the causal agents of potato late blight, chestnut blight and sudden oak death (Anderson *et al.* 2004). For most of these diseases, the source of the introduced pathogen has not been identified and the evolutionary process leading to disease emergence has not been determined. The contemporary emergence of novel plant diseases via host jump is a rare phenomenon and few clear-cut examples of host jumps exist (Antonovics *et al.* 2002; Perez *et al.* 2008; Silva *et al.* 2012). However, phylogenetic analyses of both host and pathogen for several pathosystems have revealed clear patterns of host jumps over longer evolutionary timescales (Refregier *et al.* 2008; Van der Merwe *et al.* 2008) and provided little evidence for co-speciation of pathogen and host (De Vienne *et al.* 2013).

Puccinia psidii Winter is a biotrophic fungus (Basidiomycota, Uredinales) that is the causal agent of rust diseases commonly referred to as eucalypt, guava, 'ohi'a, or myrtle rust. The fungus is macrocyclic and autoecious but does not produce the haploid pycnial or spermogonial stage that is responsible for fertilization and re-establishment of the dikaryon in other macrocyclic rusts (Glen *et al.* 2007). Teliospores and basidiospores can sometimes be observed under favourable conditions but are rare and their significance in the life cycle is unclear (Glen *et al.* 2007). It is thought that *P. psidii* reproduces largely clonally through urediniospores, but many questions remain concerning the life cycle and mating system of this pathogen. *Puccinia psidii* is an unusual rust in that it exhibits a wide host range and is known to infect over 33 plant genera and 129 species, primarily in the Myrtaceae (Tommerup *et al.* 2003; Carnegie & Lidbetter 2012; Farr & Rossman 2012). Despite the economic importance of *P. psidii* as a pathogen of eucalypts in Brazil and its newly emerging status as a threat to myrtaceous species worldwide (Coutinho *et al.* 1998; Tommerup *et al.* 2003; Glen *et al.* 2007; Carnegie & Lidbetter 2012), little is known about the evolutionary history of the fungus including its origin. The pathogen was first described on guava (*Psidium guajava* L.) in southern Brazil in 1884 (Winter 1884; MacLachlan 1938), hence the species epithet '*psidii*' reflecting the generic name of guava. Following the introduction of eucalypts (*Eucalyptus* spp.) to Brazil, first as specimens in botanic gardens in 1825, and then with the establishment of commercial plantations in the early 1900s (Doughty 2000), a rust pathogen was found infecting eucalypts in 1912 and reported in 1944 (Gonçalves 1929; Joffily 1944; Coutinho *et al.* 1998). The causal agents of guava and eucalypt rust appear morphologically identical and thus have been considered conspecific (Joffily 1944). The initial description of the pathogen from guava, the timing of the first appearance of the rust disease on eucalypts, and the documented wide host range of the pathogen gave support to a widely held hypothesis that *P. psidii* jumped from guava to eucalypts shortly after the introduction of eucalypts to Brazil (Castro *et al.* 1983); however, this hypothesis has never been tested critically. *Puccinia psidii* is considered endemic to Brazil (Tommerup *et al.* 2003) but has also been reported to infect diverse myrtaceous hosts in South and Central America, the Caribbean, Mexico, USA (Florida, California, Hawaii), Japan, China, Australia and South Africa (Laundon & Waterston 1965; Marlatt & Kimbrough 1979; Kawanishi *et al.* 2009; Carnegie *et al.* 2010; Zhuang & Wei 2011; Carnegie & Lidbetter 2012; Roux *et al.* 2013). Currently, *P. psidii* 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) and represents an existing and emerging threat to native and exotic myrtaceous species worldwide (Coutinho *et al.* 1998; Uchida *et al.* 2006; Glen *et al.* 2007; Carnegie & Lidbetter 2012).

Artificial inoculation studies of *P. psidii* sampled from different hosts have provided evidence for physiologic or pathogenic variation in populations of the pathogen (MacLachlan 1938; Ferreira 1981; Castro *et al.* 1983; Coutinho & Figueiredo 1984; Coelho *et al.* 2001; Aparecido *et al.* 2003), but these studies have not provided definitive clues as to the source of the pathogen infecting eucalypts in Brazil. Ferreira (1981) demonstrated that the pathogen from guava was incapable of infecting eucalypts or rose apple (*Syzygium jambos* (L.) Alston). Using pathogen samples from eucalypts, guava and rose apple, Coelho *et al.* (2001) found evidence for three pathogenic groups including one that infected eucalypts/rose apple, another that infected eucalypts/guava, and a third that infected only guava. In contrast, Aparecido *et al.* (2003) identified four pathogenic groups of *P. psidii* in Brazil including a rose apple group, a eucalypts group, a jabuticaba (*Myrciaria cauliflora* (Mart.) O. Berg) group, and a fourth group from cambucá (*Plinia edulis* (Vell.) Sobral). Pathogenic variation within and among pathogen populations and the diverse genetic backgrounds of inoculated hosts has presented challenges for host-specificity studies with *P. psidii*. The equivocal results observed among cross-inoculation studies to date are likely due to variation in

pathogen sources, inoculation methods and conditions, and host genotypes. Although these studies strongly support the hypothesis of host specialization among populations of *P. psidii*, they are unable to predict a likely source for the introduction to eucalypts. Despite several reports of pathogenic variability in *P. psidii*, there has been only limited application of molecular markers to indirectly assess host specificity and population structure of this pathogen (Zhong *et al*. 2008, 2011). The objective of this study was to characterize the genetic diversity of *P. psidii* across a diverse range of hosts in Brazil and Uruguay and test the hypothesis that eucalypt rust in Brazil was derived via host jump of the pathogen from guava to eucalypts. To critically test this hypothesis, *P. psidii* populations were sampled from eucalypts, guava, and five other myrtaceous hosts throughout southern Brazil and Uruguay, and microsatellite markers were used to determine the population structure of *P. psidii* infecting myrtaceous hosts in Brazil.

Materials and methods

Sampling

Single uredinial pustules of *P. psidii* ca. 6 mm diameter were collected between March 2008 and August 2009 from 148 individual plants representing seven myrtaceous taxa. These taxa included eucalypts, guava, rose apple, Brazilian guava (*Psidium guineense* Swartz), Java plum (*Syzygium cumini* (L.) Skeels), jabuticaba and pitanga (*Eugenia uniflora* L.) in nine Brazilian states and one location in Uruguay (Fig. 1, Table S1, Supporting information). Because *P. psidii* infects only young tissues and disease development is highly dependent on environmental conditions, the number of samples collected from each state and host varied due to microclimatic conditions, host prevalence, and the phenological state of host organs. All sampled hosts were georeferenced.

Microsatellite genotyping

Samples were genotyped at 10 microsatellite loci (PpSSR012, PpSSR014, PpSSR018, PpSSR022, PpSSR087, PpSSR102, PpSSR146, PpSSR161, PpSSR178 and PpSSR 195) originally developed from a genomic DNA library of *P. psidii* strain ShaoR14 enriched for microsatellite motif AG (Zhong *et al*. 2008; Table S2, Supporting information). To improve amplification and scoring of the loci PpSSR022, PpSSR087, PpSSR102, PpSSR146, PpSSR178 and PpSSR195, new primers (Table S2) were designed based on *P. psidii* microsatellite sequences deposited in GenBank (Zhong *et al*. 2008) using Primer3 (Rozen & Skaletsky 2000; http://primer3.ut.ee/). Genomic DNA was extracted directly from each *P. psidii* pustule (containing fungal and host tissue) using a modified CTAB-based protocol (Doyle & Doyle 1987). PCR mixtures included 10× PCR buffer II (Bioline USA, Inc, Taunton, MA, USA), 2.5 mM $MgCl_2$, 500 μM dNTPs, 75 nM forward (labelled with either FAM or HEX fluorescent dye) and reverse primers, 1 unit Taq DNA polymerase (Bioline), and 30 ng DNA template in 20 μl reaction volumes. 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, 40–55 °C (depending on the locus) for 30 s, 72 °C for 80 s, 35 cycles at 94 °C for 15 s, 40–55 °C (depending on the locus) for 15 s and 45 s at 72 °C, followed by a final extension period of 72 °C for 5 min, ending with a 4 °C hold. Fragment analysis was conducted at the University of Wisconsin Biotechnology Center (http://www.biotech.wisc.edu) via capillary electrophoresis using an ABI 3700 DNA automated sequencer (Life Technologies Corporation, Carlsbad, CA, USA). Positive and negative controls were included for each locus scored, and scoring was repeated for representative alleles for each locus to ensure the accuracy of genotyping. Allele sizes were estimated using marker standards (ROX Geneflo 625; CHIMERx, Milwaukee, WI, USA) and scored using ABI PeakScanner Analysis Software v1.0 (Life Technologies).

Data analysis

Genetic diversity and population structure. Null alleles were identified using MICROCHECKER 2.2.3 (Oosterhout *et al*. 2004). Population genetic analyses of all samples grouped by host (hereafter referred to as 'population') included the average number of alleles per locus, average number of effective alleles per locus, Shannon Index of allelic diversity, number of private alleles, observed heterozygosity, expected heterozygosity, fixation index and number of multilocus genotypes estimated in GenAlEx 6.4 (Peakall & Smouse 2006). Allelic diversities of the eucalypt- and guava-associated populations were estimated using the Shannon Index of allelic diversity (Sherwin *et al*. 2006), and the difference in these indices between the eucalypt- and guava-associated populations was tested using 10 000 random permutations of the data set in GeneAlEx. Clonal diversities of the eucalypt- and guava-associated populations were estimated using the Shannon Index of clonal diversity without sample size correction in GenoDive v2.0b22 (Meirmans & Van Tienderen 2004). The difference in clonal diversities between the eucalypt- and guava-associated populations was tested using 10 000 bootstrapped data sets with subsampling to match population sizes in GenoDive. Observed clonal diversities and expected clonal diversities under random mating were estimated for the eucalypt- and

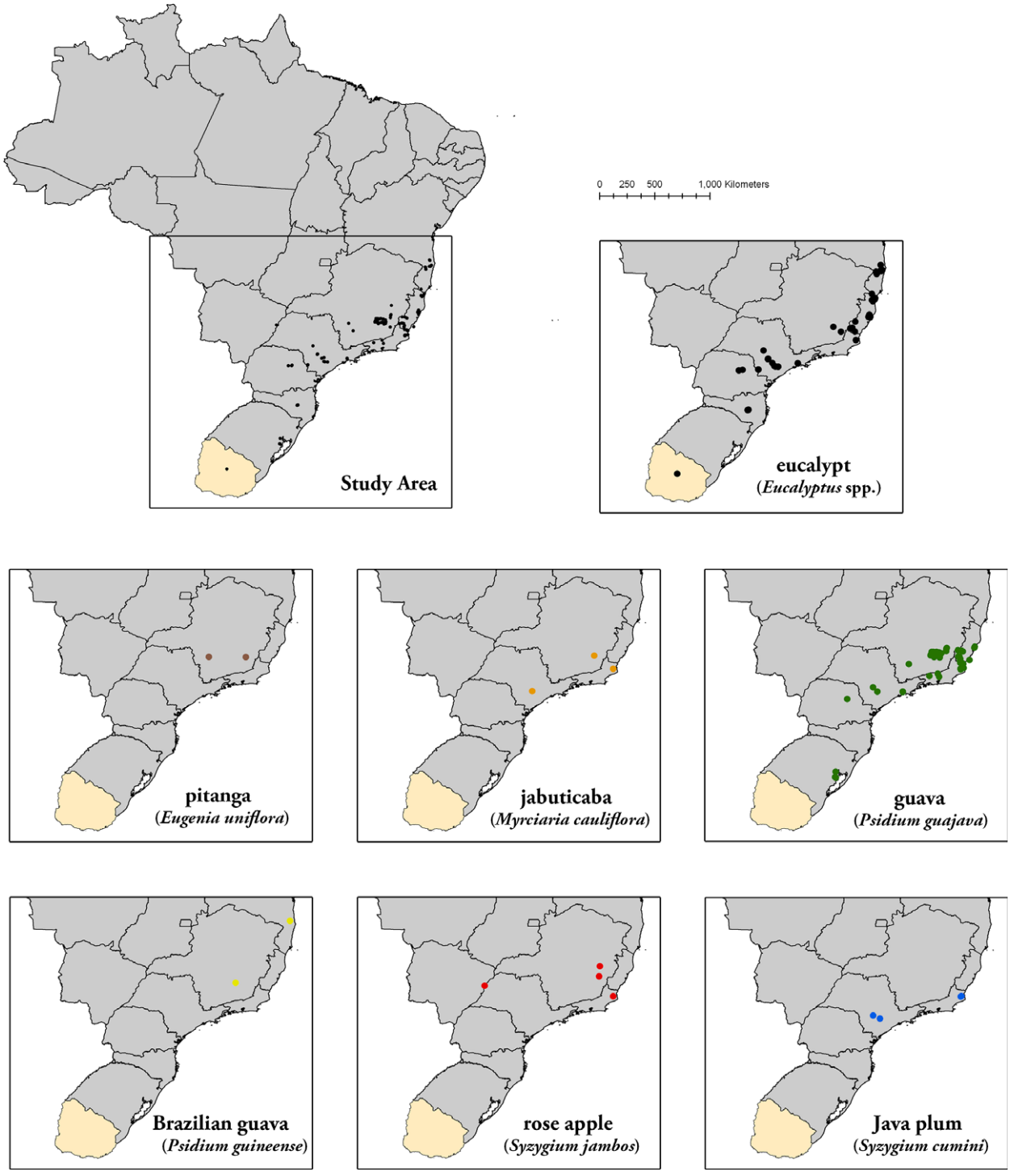

Fig. 1 Sampling locations of *Puccinia psidii* obtained from seven myrtaceous hosts in Brazil and *Eucalyptus* spp. in one location in Uruguay (shaded in yellow). Isolates associated with eucalypts (*Eucalyptus* spp.) are indicated in black, pitanga (*Eugenia uniflora*) in brown, jabuticaba (*Myrciaria cauliflora*) in orange, guava (*Psidium guajava*) in green, Brazilian guava (*Psidium guineense*) in yellow, rose apple (*Syzygium jambos*) in red and Java plum (*Syzygium cumini*) in blue.

guava-associated populations in GenoDive and compared within each population using 10 000 random permutations of the data set to simulate random mating. Genotypic evenness was estimated by dividing the effective number of genotypes by the total number of genotypes for each of the two host-associated populations in GenoDive.

The probabilities that multiple occurrences of the same multilocus genotype (MLG) in the eucalypt-associated and guava-associated *P. psidii* populations were the result of independent sexual reproductive events rather than clonal reproduction were estimated using the p_{sex} approach of Parks & Werth (1993) as implemented in MLGsim2.0 (http://www.rug.nl/research/theoretical--biology/downloads), a revised version of MLGsim1.0 (Stenberg *et al.* 2003). This analysis provides a test for the overrepresentation of MLGs as well as the power of the microsatellite markers to discriminate clonally reproduced MLGs. P_{sex} values were obtained using allele frequencies estimated using the 'round robin' approach (Parks & Werth 1993) with clone-corrected data sets and corrected for inbreeding observed at each locus (Arnaud-Haond *et al.* 2007). P_{sex} values were tested statistically using Monte Carlo simulations of p_{sex} values under the null hypothesis of random mating implemented in MLGsim2.0. Ten thousand p_{sex} values were simulated, and this distribution was used to estimate a *P*-value for each p_{sex} value estimated for each MLG in each population.

Sampled isolates were assigned to genetic clusters using a Bayesian genetic clustering algorithm implemented in STRUCTURE v2.3.4 (Pritchard *et al.* 2000). The pathogen was sampled from seven myrtaceous hosts and specific MLGs were highly host-associated so posterior probabilities were estimated for $K = 1$ to $K = 7$ to reflect the number of hosts sampled. We assumed an admixture model, correlated allele frequencies and geographic location of sampling was not used in clustering. STRUCTURE analyses were performed with the complete data set ($n = 148$) that included replicate MLGs (i.e. nonclone corrected). Additional runs to estimate the sensitivity of results to model choice using a nonadmixture model and uncorrelated allele frequencies did not change results. Fifty thousand burn-in generations were employed for each of 10 replicate runs of 1 000 000 generations of the MCMC sampler for each *K*. The optimal value of *K* was inferred using the method of Evanno *et al.* (2005) implemented in STRUCTURE HARVESTER web v0.6.93 (Earl & von Holdt 2012). Results from multiple runs for each *K* were collated in CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007), and the output visualized in DISTRUCT v1.1 (Rosenberg 2004).

Principal coordinates analysis (PCoA), based on a covariance matrix with data standardization (Smouse & Peakall 1999), was performed using GenAlEx 6.4 (Peakall & Smouse 2006). The data set used for PCoA consisted of unique multilocus haplotypes ($n = 25$). To examine the relationships among MLGs, a minimum-spanning network was estimated using the genetic distance measure of Bruvo *et al.* (2004), which employs a stepwise mutation model. A distance of 0.04 was equivalent to one mutational step (one repeat) but larger distances do not strictly correspond to a given number of mutational steps. All tied trees were included in the network, which was visualized using HapStar (Teacher & Griffiths 2011) and modified for presentation in Inkscape v0.47 (Bah 2011).

Evolutionary scenarios tested with approximate Bayesian computation. The posterior probabilities of various evolutionary scenarios generating the patterns of genetic variation observed among sampled isolates were estimated using approximate Bayesian computation implemented in DIYABC v1.0.4.46 (Cornuet *et al.* 2010). Invasion scenarios focused on evolutionary relationships among 2 or 3 genetic clusters inferred with highest likelihoods in the STRUCTURE analyses with or without an additional unsampled or 'ghost' population (Guillemaud *et al.* 2010). Given the hypothesis that guava was the source of the introduction to eucalypts, the major evolutionary scenario of interest was that of *P. psidii* expanding its host range to eucalypts from guava within the past 200 years. This scenario was compared other evolutionary scenarios where pathogen populations sampled from eucalypts, guava and other myrtaceous hosts coalesced further back in time and/or scenarios where eucalypt populations coalesced with a ghost population (Fig. 2). Assuming two genetic populations, Scenario 1 considered the eucalypt population coalescing with the guava population at time t_1 and with a ghost population at t_2 while Scenario 2 assumed that the eucalypt population coalesced with a ghost population at t_1 followed by the guava and ghost populations coalescing at time t_2 (Fig. 2). Assuming three genetic populations, Scenario 3 considered the eucalypt population coalescing with the guava population at t_1 and with the 'other' population (isolates sampled from jabuticaba, java plum, and pitanga) at t_2 (Fig. 2). Scenario 4 evaluated the probability of the eucalypt population coalescing with the other population at time t_1 and with the guava population at t_2. Scenario 5 evaluated the probability of the eucalypt, guava and other populations coalescing at t_2, while Scenario 6 evaluated the probability of the eucalypt population coalescing with a ghost population at t_1 followed by coalescence of the guava and other population at t_2 (Fig. 2). All one- and two-population summary statistics were estimated for each simulation and default settings for microsatellite locus mutational parameters were used except for locus 513. The allele size range observed at this locus was considerably greater than that observed for other loci (126 bp), so we extended the size range parameter for this locus from the default value of 40 to 160. Three or four effective population size parameters and two time parameters were estimated for all scenarios. A constraint was placed on the time parameters so that t_2

was always greater (older) than t_1. Prior uniform distributions for t_1 were set to an interval of 1–1000 years and t_2 were set to 1–100 000 years for all simulations. We had no meaningful estimates of effective population sizes so default uniform prior distributions (10–10 000) were used with no constraints. To estimate the effect of clonal reproduction on the ABC analyses, all simulations were run with the entire data set (n = 148) and with a clone-corrected data set consisting of 25 unique MLGs. One million data sets were simulated for each scenario, and posterior probabilities of parameter estimates were estimated using logistic regression using 1% of the simulated data sets closest to the observed data (Cornuet *et al.* 2008). Confidence in model choice was evaluated by simulating data sets under each scenario using parameters estimated from the posterior distributions. Type I error rates were estimated by simulating 500 data sets for each tested scenario and counting the proportion of data sets simulated under the best scenario that resulted in highest posterior probabilities for other scenarios. Type II error rates were estimated as the proportion of 500 simulated data sets that resulted in highest posterior probability for the best scenario when simulated under each of the competing scenarios.

Results

Genetic diversity and population structure

Ten microsatellite loci were scored for 148 *P. psidii* samples derived from seven hosts located in nine Brazilian states and Uruguay (Table S1, Supporting information). All loci were polymorphic with no evidence of null alleles (Table 1), and two to seven alleles were detected per locus across the entire sample set (Table S2, Supporting information). Average and effective number of alleles per locus varied from a low of 1.3 and 1.3, respectively, for the pitanga-associated population to highs of 2.1 and 1.8, respectively, for the eucalypt-associated population (Table 1). The number of private alleles ranged from 0 for the guava-associated population to 8 for the jabuticaba-associated population. Shannon indices of allelic diversity for the eucalypt- and guava-associated populations were 0.536 and 0.384, respectively (P = 0.0100; Table 1). Observed and expected heterozygosities were higher in the eucalypt-associated population compared with the guava-associated population and both had strongly negative fixation indices (F_{IS} = −0.775 to −0.719) indicating an excess of heterozygotes (Table 1). Clonal diversity was higher in the eucalypt-associated population compared to the guava-associated population (Table 1), and this difference was highly significant when tested with bootstrapping ($P < 0.0001$). Observed clonal diversities were significantly less ($P < 0.0001$) than expected clonal diversities under random mating for both the eucalypt-associated and guava-associated *P. psidii* populations (Table 1). The hypothesis that multiple occurrences of the same multilocus genotype (MLG) was due to independent sexual reproductive events was rejected ($P < 0.05$) for 6 of 11 recurrent MLGs in the eucalypt-associated *P. psidii* population (Table S3, Supporting information). In this same population, we failed to reject ($P > 0.05$) the hypothesis for 5 MLGs detected 2 or 3 times (Table S3, Supporting information). The hypothe-

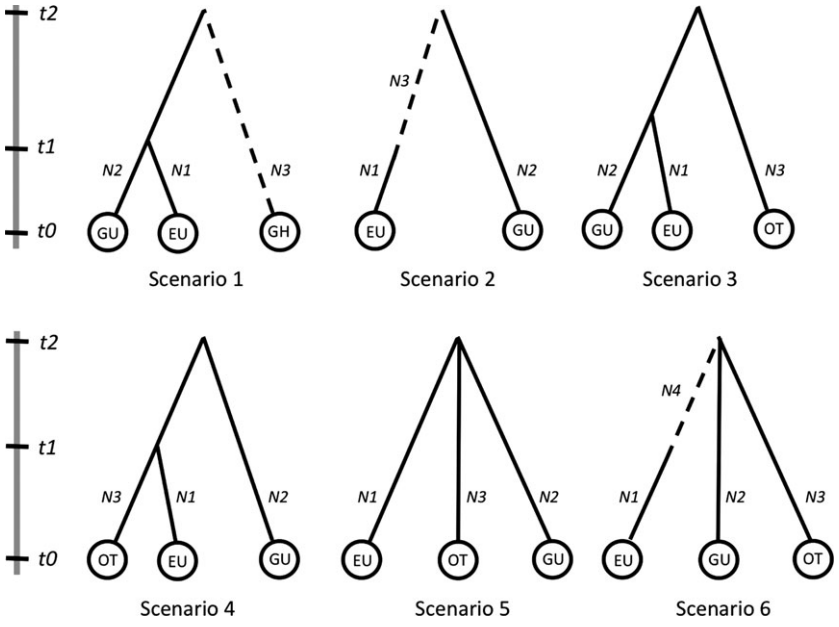

Fig. 2 Graphical representation of six evolutionary scenarios modelled in DIYABC. Scenarios 1 and 2 assumed two genetic populations and an unsampled (ghost) population as inferred with highest likelihood in the STRUCTURE analyses using the Evanno *et al.* (2005) method. Scenarios 4–6 assumed three genetic populations with or without a ghost population. Parameters estimated included divergence time (t) and effective population size (N). Population labels are guava (GU), eucalypts (EU), 'other' (jabuticaba, Java plum and pitanga; OT) and unsampled or 'ghost' (GH). Solid lines indicate extant sampled populations while dotted lines indicate ghost populations. Timescale is at left and time parameter t_2 was forced to be greater than t_1 in all analyses.

Table 1 Summary of allelic and genotypic variation in populations of *Puccinia psidii* sampled from seven myrtaceous hosts in Brazil and eucalypt in Uruguay

	Host						
	Eucalypt (*Eucalyptus* spp.)	Guava (*Psidium guajava*)	Rose apple (*Syzygium jambos*)	Brazilian guava (*Psidium guineense*)	Java plum (*Syzygium cumini*)	Jabuticaba (*Myrciaria cauliflora*)	Pitanga (*Eugenia uniflora*)
Sample size	70	63	4	2	4	3	2
Alleles per locus	2.100 (0.314)*	1.700 (0.300)	1.600 (0.163)	1.600 (0.163)	1.700 (0.153)	1.800 (0.133)	1.300 (0.153)
Effective alleles per locus	1.754 (0.191)	1.625 (0.297)	1.600 (0.163)	1.600 (0.163)	1.700 (0.153)	1.800 (0.133)	1.300 (0.153)
Private Alleles	2	0	1	1	5	8	4
Allelic Diversity†	0.536	0.384	—‡	—	—	—	—
Number of multilocus genotypes	15	5	1	1	1	1	1
Observed heterozygosity	0.630	0.425	—	—	—	—	—
Expected heterozygosity	0.355	0.247	—	—	—	—	—
Fixation index	−0.775	−0.719	—	—	—	—	—
Clonal diversity – observed§	0.993	0.570	—	—	—	—	—
Clonal diversity – expected¶	1.834	1.739					
Genotypic evenness**	0.384	0.650	—	—	—	—	—

*Number in parentheses = standard error.
†Shannon information index of allelic diversity (Sherwin *et al.* 2006).
‡Not estimated due to limited sample size.
§Shannon information index of clonal diversity – observed (Meirmans & Van Tienderen 2004).
¶Shannon information index of clonal diversity – expected under random mating (Meirmans & Van Tienderen 2004).
**Effective number of genotypes/total number of genotypes.

sis of multiple occurrences of the same MLG due to independent sexual reproductive events was strongly rejected ($P < 0.01$) for all 4 recurrent MLGs in the guava-associated *P. psidii* population (Table S3, Supporting information).

Results of the STRUCTURE analyses indicated that ln likelihoods of the data plateaued quickly from $K = 2$ to $K = 10$ (Fig. S1, Supporting information) and $K = 2$ was selected as the best estimate of the number of genetic clusters using the Evanno *et al.* (2005) method. For $K = 2$, isolates sampled from eucalypts and guava were assigned to different clusters, labelled in blue and red, respectively (Fig. 3). Isolates from rose apple, Java plum, jabuticaba and pitanga were assigned to the eucalypt cluster, while isolates from Brazilian guava were assigned to the guava cluster (Fig. 3). For $K = 3$, isolates from eucalypts and guava were similarly assigned to different clusters (Fig. 3). Rose apple isolates were again assigned to the eucalypt cluster and isolates from Brazilian guava were assigned to the guava cluster. Isolates from the remaining 3 myrtaceous hosts (jabuticaba, Java plum, pitanga) were assigned to a third cluster (Fig. 3). For all higher values of K evaluated, isolates sampled from eucalypts and guava were consistently assigned to different genetic clusters (Fig. 3). Twenty-five MLGs were identified (Table 1), and each MLG was completely correlated with host taxon with no MLGs shared among populations from different hosts. The largest numbers of MLGs were associated with eucalypt and guava, with 15 and 5, respectively, and a single, distinct MLG was associated with each of the remaining five hosts (Brazilian guava, rose apple, Java plum, jabuticaba and pitanga; Table 1).

Principal coordinates analysis (PCoA) revealed a high degree of genetic differentiation among MLGs derived from different hosts (Fig. 4) and supported the STRUCTURE analyses. In some cases, individuals derived from the same host separated by more than 1000 km shared the same MLG, while samples collected less than 1 km apart from different hosts had different

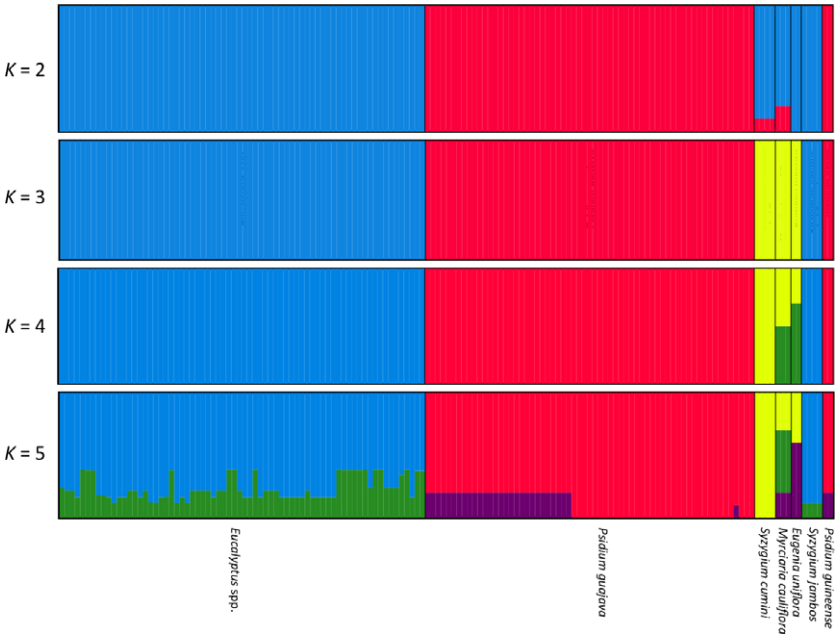

Fig. 3 Population structure of 148 *Puccinia psidii* isolates sampled from seven myrtaceous hosts in Brazil inferred using a Bayesian clustering algorithm implemented in STRUCTURE v2.3.4. Each individual isolate is represented by a vertical line partitioned into shaded segments corresponding to the isolate's estimated mean membership coefficient in $K = 2$ to $K = 5$ genetic clusters. Membership coefficients were estimated from 10 replicate runs for each K. Vertical black lines separate isolates sampled from different hosts and each host is labelled below.

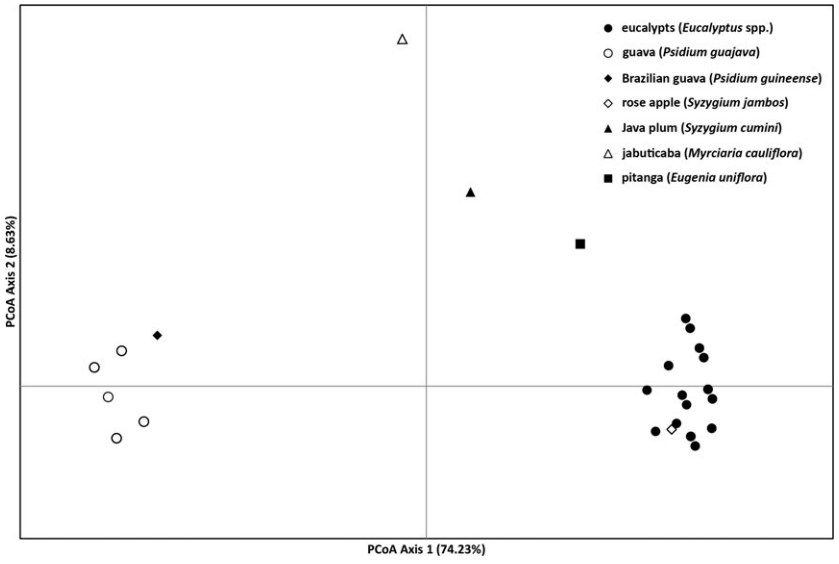

Fig. 4 Principal coordinates analysis of unique microsatellite multilocus genotypes ($n = 25$) observed among 148 *Puccinia psidii* isolates sampled from different hosts throughout Brazil and eucalypts in Uruguay. Coordinates are based on a covariance matrix with data standardization and the first two axes explain 83% of the observed variation. *P. psidii* associated with eucalypts (*Eucalyptus* spp.) indicated by black circles, pitanga (*Eugenia uniflora*) by black squares, jabuticaba (*Myrciaria cauliflora*) by white triangles, guava (*Psidium guajava*) by white circles, Brazilian guava (*Psidium guineense*) by black diamonds, rose apple (*Syzygium jambos*) by white diamonds and Java plum (*Syzygium cumini*) by black triangles.

MLGs. Two major clusters emerged in the PCoA including a cluster formed by MLGs associated with eucalypt and rose apple and another cluster formed by MLGs associated with guava and Brazilian guava (Fig. 4). Three unique MLGs were each associated with Java plum, jabuticaba and pitanga (Fig. 4). The first two axes explained 83% of total variation, with the first axis separating guava-/Brazilian guava-associated MLGs from the eucalypt-/rose apple-associated MLGs and explaining 74% of the variation. The second axis separated the jabuticaba-, Java plum- and pitanga-associated MLGs from the two major clusters and explained 9% of the variation.

The minimum-spanning network was highly congruent with the PCoA and STRUCTURE analyses revealing five distinct subnetworks with each associated with one or two hosts (Fig. 5). Each subnetwork was separated by a large genetic distance (Bruvo distance = 0.41–0.69) from its neighbour and represented allelic differences at multiple microsatellite loci (Fig. 5). The largest subnetwork comprised 15 MLGs associated with eucalypts (MLGs 1–15) as well as a single MLG (MLG 21) associated with rose apple. Most of the connections between MLGs in this subnetwork represented single mutational steps with the exception of connections between MLG 1 and 4 (5 mutational steps at locus 501) and MLG 14

and 21 (17 mutational steps at locus 510). The second largest subnetwork comprised 5 MLGs (MLG 16–20) associated with guava and a single MLG (MLG 22) associated with Brazilian guava (Fig. 5). This subnetwork was separated from the eucalypt-/rose apple-associated MLG network by a genetic distance of 0.51 representing multiple mutational steps at all but two of the 10 loci scored. Larger distances were common within this subnetwork with all but one connection representing multiple mutational steps. The remaining three subnetworks each consisted of a single MLG, each associated with a different host (Java plum-associated MLG 23, jabuticaba-associated MLG 24, and pitanga-associated MLG 25). Among the 15 MLGs associated with eucalypts, only two (MLG 14 and 9) were frequent (>10%), with frequencies of 31 and 23%, respectively (Fig. 5). Among guava-associated MLGs, all but one (MLG 16) were frequent and these MLGs were more evenly distributed with MLG 17, 18, 19 and 20 exhibiting

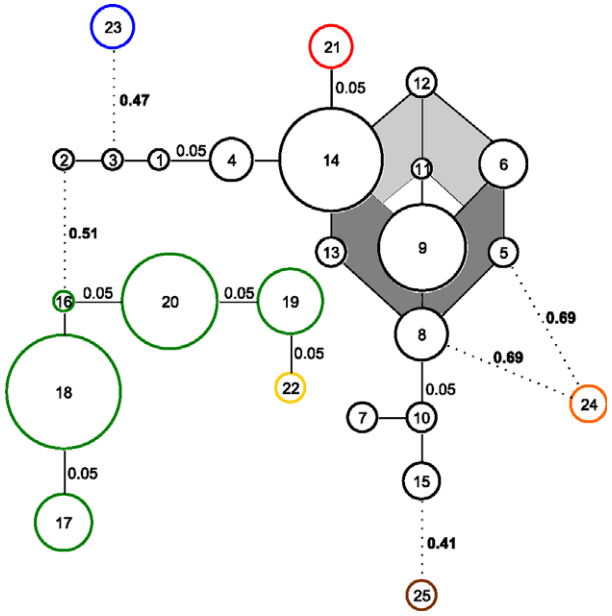

Fig. 5 Minimum-spanning network of *Puccinia psidii* microsatellite multilocus genotypes (MLGs) sampled from seven myrtaceous hosts in Brazil and eucalypts in Uruguay. MLGs are represented by black circles for the *P. psidii* population from eucalypts (*Eucalyptus* spp.), green from guava (*Psidium guajava*), red from rose apple (*Syzygium jambos*), yellow from Brazilian guava (*Psidium guineense*), blue from Java plum (*Syzygium cumini*), orange from jabuticaba (*Myrciaria cauliflora*) and brown from pitanga (*Eugenia uniflora*). Sizes of circles are proportional to MLG frequency and each MLG is numbered. Connections are labelled with Bruvo genetic distances if different from 0.04, which corresponds to 1 mutational step at one locus. Broken lines connect MLGs that differ by distances greater than 0.40. Loops in the network (i.e. within the eucalypt-associated MLGs) indicate multiple-tied minimum-spanning trees.

frequencies of 11, 43, 14, and 30%, respectively (Fig. 5). The more even distribution of guava-associated MLGs is reflected in a higher genotypic evenness index compared with the eucalypt-associated MLGs (Table 1).

Evolutionary scenarios tested with approximate Bayesian computation

Approximate Bayesian computation (ABC) analyses assuming two or three genetic populations consistently revealed that evolutionary scenarios involving a divergence event between guava- and eucalypt-associated populations within the past 1000 years to have very low posterior probabilities compared to alternative scenarios (Table 2). Assuming two genetic populations, Scenario 2 (older divergence event between guava- and eucalypt-associated populations) had significantly higher posterior probability that Scenario 1 (more recent divergence event between guava- and eucalypt-associated populations) for both the nonclone-corrected and clone-corrected data sets (Table 2). Assuming three genetic populations, Scenario 4 (recent divergence event between eucalypt-associated population and the 'other' population) had significantly higher posterior probabilities than Scenario 3 (more recent divergence event between guava- and eucalypt-associated populations) for both nonclone-corrected and clone-corrected data sets (Table 2). Scenarios 5 and 6 where three or four populations diverged further back in time had lower posterior probabilities compared with Scenario 4 but higher posterior probabilities than Scenario 3 for both data sets. Type I and Type II error rates were low ($P < 0.05$) for both non clone-corrected and clone-corrected data sets assuming either 2 or 3 genetic populations indicating high confidence in the chosen scenarios (Table S4, Supporting information).

Discussion

Populations of the rust pathogen *P. psidii* infecting seven Myrtaceous species in south-eastern Brazil were strongly genetically differentiated by host, providing the first genetic evidence for host-associated multilocus genotypes (MLGs) or biotypes of this pathogen. Lack of gene flow among host-associated genotypes was supported by the observation of private alleles in most populations and by the presence of unique MLGs associated with specific hosts spanning large geographic distances in Brazil. Thus, the genetic structure of *P. psidii* populations in Brazil appears to be strongly influenced by host. The eucalypt-associated population of *P. psidii* was more genetically and genotypically diverse than the guava-associated population, and these populations were highly genetically differentiated. Principal coordinates and Bayesian clustering analyses provided

Table 2 Posterior probabilities of evolutionary scenarios for emergence of *Puccinia psidii* on eucalyptus in Brazil

Populations	Clone correction*	Scenario†	Posterior probability‡	95% Confidence interval
2	No	1	0.0068	0.0000, 0.0172
		2	0.9932	0.9828, 1.0000
	Yes	1	0.0371	0.0000, 0.1079
		2	0.9629	0.9732, 0.9941
3	No	3	0.0000	0.0000, 0.0000
		4	0.6523	0.4381, 0.8664
		5	0.1338	0.0472, 0.2204
		6	0.2140	0.0786, 0.3494
	Yes	3	0.0000	0.0000, 0.0000
		4	0.9836	0.9732, 0.9941
		5	0.0069	0.0023, 0.0115
		6	0.0094	0.0033, 0.0156

*Analysis performed with entire data set (n = 148) including replicate clonal genotypes (non clone-corrected) or with a reduced data set (n = 25) where multiple occurrences of each multilocus genotype were censored (clone-corrected).
†Evolutionary scenarios illustrated in Fig. 2.
‡Posterior probabilities estimated using logistic regression of 1% of the simulated data sets closest to the actual data set.

evidence for two genetic clusters, with isolates sampled from eucalypts and guava consistently assigned to different clusters. No MLG was shared by the eucalypt- and guava-associated populations and both the principal coordinates analysis and minimum-spanning network revealed large genetic distances between MLGs associated with each of these hosts. Multiple mutational steps were observed at eight of 10 microsatellite loci between MLGs associated with eucalypts versus guava. Our results support the existence of at least two distinct biotypes, where biotype is defined as a group of organisms with similar genotypes. In this case, these genetically differentiated populations have unique host associations, one associated with eucalypt and the other with guava. Further research is required to determine whether these biotypes represent distinct *forma speciales* or possibly emerging species as exemplified by *Colletotrichum kahawae* (Silva *et al.* 2012). Approximate Bayesian analyses indicated that divergence between guava- and eucalypt-associated populations within the past 1000 years is highly unlikely and allow us to strongly reject the hypothesis that guava was the source of *P. psidii* that currently infects eucalypts in Brazil, as eucalypts were first introduced to Brazil less than 185 years ago (Doughty 2000).

Two pairs of closely related MLGs were associated with two *Psidium* species, guava and Brazilian guava, and another pair with eucalypts and rose apple. Additional unique MLGs were associated with each of Java plum, jabuticaba and pitanga. Despite the small sample sizes, only a single MLG was associated with each host even though the pathogen was sampled from plants separated by large geographic distances (Fig. 1). These results support previous studies of host specialization in *P. psidii* (MacLachlan 1938; Joffily 1944; Ferreira 1981; Castro *et al.* 1983; Coutinho & Figueiredo 1984; Coelho *et al.* 2001; Aparecido *et al.* 2003) despite the documented wide host range of *P. psidii* (Farr & Rossman 2012). Of further interest, these results from Brazil also contrast with recent observations from Hawaii and Australia where a single MLG (distinct from the MLGs found in Brazil) is capable of infecting multiple myrtaceous species (Loope 2010; Zhong *et al.* 2011; Carnegie & Lidbetter 2012). Our current concept of *P. psidii* is based on morphology, but it is evident that additional study is required to better assess the host specificity and environmental requirements of particular biotypes. Our analyses clearly show that genetic variation among *P. psidii* populations warrants greater consideration for more accurate assessments of critical invasive risks associated with introduction of *P. psidii* to ecologically diverse global regions where myrtaceaous hosts occur (Loope & Uchida 2012).

Populations of *P. psidii* sampled from all hosts exhibited highly clonal structures as evidenced by the overrepresentation of multilocus genotypes, highly negative fixation indices, and lack of reticulation throughout most of the minimum-spanning network. Such results are hallmarks of asexually reproducing populations (Arnaud-Haond *et al.* 2007; Goyeau *et al.* 2007) and suggest a lack of regular meioses in the life cycle of *P. psidii*. Field observations of infected hosts have indicated that reproduction occurs mainly through the production of asexual urediniospores (AC Alfenas, *unpublished*, Glen *et al.* 2007). Strongly clonal populations were also observed for the wheat leaf rust pathogen in France (Goyeau *et al.* 2007), a fungus with similar biology to *P. psidii* and which similarly lacks a known sexual

cycle. In most rust fungi, karyogamy and meiosis occur in teliospores resulting in the production of haploid, recombinant basidiospores that infect and re-establish the dikaryon. As *P. psidii* teliospores and basidiospores are rarely observed on any myrtaceous host (A. C. Alfenas, unpublished), the significance of these stages in the life cycle of the pathogen is uncertain. The minimum-spanning network revealed six loops in the eucalypt-associated population that could be explained by recombination or by parallel mutations. Under an infinite alleles model, parallel mutations are considered highly unlikely and recombination is the favoured hypothesis to explain the occurrence of 4 different haplotypes at 2 loci (Hudson & Kaplan 1985). As the probability of parallel and/or back mutations may be significantly greater for microsatellites compared with other types of markers, it is possible that the loops we observed in the minimum-spanning network were caused by parallel mutations rather than by recombination. Further study of the mating system of *P. psidii* that includes the application of alternative marker systems such as SNPs may allow this hypothesis to be tested.

Processes underlying biological invasion and disease emergence are of great interest as anthropogenic activities continue to eliminate barriers to pathogen dispersal and change the environment in ways that threaten human health, food security and biological conservation (e.g. Santini *et al.* 2013). Examples of emerging infectious diseases of woody plants as a result of pathogen introduction include Dutch elm disease caused by *Ophiostoma* spp. (Brasier 2001), chestnut blight caused by *Cryphonectria parastica* (Anagnostakis 1987), dogwood anthracnose caused by *Discula destructiva* (Carr & Banas 2000), pitch canker caused by *Fusarium circinatum* (Gordon *et al.* 2001), white pine blister rust caused by *Cronartium ribicola* (Kinloch 2003), and sudden oak death caused by *Phytophthora ramorum* (Grünwald *et al.* 2012). Giraud *et al.* (2010) propose that the emergence of new fungal plant diseases may be the result of ecological speciation. Host jumps have undoubtedly been responsible for the emergence of several devastating diseases in plants and animals (Woolhouse *et al.* 2005) and while not all such host jumps have led to ecological speciation, there are documented cases where novel host associations have served as powerful drivers of divergent selection ultimately leading to speciation (e.g. Silva *et al.* 2012). It is extremely difficult to predict disease emergence and host risk as host jumps depend on complex and interacting demographic, ecological and environmental factors (Barrett & Heil 2012).

Although we can confidently reject the guava to eucalypt transmission hypothesis for *P. psidii*, a critical question remains concerning the source of *P. psidii* that infects eucalypts in Brazil. Tracking the origin of introduced pathogens has been problematic (e.g. Stukenbrock & McDonald 2008; Grünwald *et al.* 2012). Comprehensive global surveys are needed to identify potential sources of eucalypt-associated *P. psidii* in Brazil. This will require sampling and genotyping of many additional pathogen samples collected globally wherever *P. psidii* occurs. Although our samples from hosts other than eucalypts and guava were limited in this study, we have no evidence to reject the hypothesis that rose apple may be the potential source of the eucalypt biotype in Brazil. The rose apple-associated MLG clustered with eucalypt-associated MLGs in the PCoA, and differed from eucalypt-associated MLG 14 by 17 mutational steps at a single locus in the network. Microsatellite loci are generally modelled using a stepwise mutation model that makes a multistep change at a microsatellite locus much less likely than a single-step change (Ohta & Kimura 1973; Bruvo *et al.* 2004). However, accumulating data from diverse microsatellite genotyping studies indicate that microsatellites may not evolve exclusively via single-step changes but may occasionally undergo larger multistep changes (Di Rienzo *et al.* 1994). Therefore, the 17-step difference at locus 510, which was observed between the rose apple-associated MLG 21 and the most common eucalypt-associated MLG, MLG 14, may have resulted from fewer than 17 individual mutational events. If we assume that locus 510 evolved in a single step, estimates of divergence time between these populations greatly decrease and may be consistent with a more recent evolutionary split between these populations since the introduction of eucalypts to Brazil. Rose apple is believed to be native to the East Indies and Malaysia and is naturalized in India, Sri Lanka, Southeast Asia, and the Pacific Islands (Morton 1987). It was introduced to Jamaica in 1762 and subsequently became established throughout much of the Caribbean and Central America. In 1825, it was brought to Hawaii from Brazil, where it had been introduced for commercial purposes in the early nineteenth century (Voeks 1997). By the late nineteenth century, the species had become widely established in West Africa, Australia and Florida, USA. In Brazil, *P. psidii* was first reported on rose apple in 1901 (Hennings 1902), just prior to its detection on eucalypts in 1912 (Gonçalves 1929; Joffily 1944; Coutinho *et al.* 1998). Additional sampling and genotyping of *P. psidii* from rose apple in its native range as well as from introduced populations and sampling from other myrtaceous hosts worldwide will be required to determine the source of the genotype infecting eucalypts.

Introductions of *P. psidii* to Brazil via imported eucalypts represent another potential source of the eucalypt biotype in Brazil, but the likelihood of this scenario is difficult to assess. Several eucalypt species from various

geographic sources (both native and non-native) have been imported numerous times to Brazil, beginning as early as 1825, with continual expansion of commercial plantings since the early 1900s (Doughty 2000). Most eucalypt germplasm imported from Australasia to Brazil has been introduced as seeds (Fonseca *et al.* 2010), which are unlikely to harbour the pathogen due to the biotrophic nature of the pathogen and limited viability of urediniospores on seed surfaces (Langrell *et al.* 2008; Lana *et al.* 2011). Further, except for the recent introduction of a distinct genotype, *P. psidii* has not been reported on native eucalypts in Australasia (Carnegie *et al.* 2010; Carnegie & Lidbetter 2012). Nevertheless, it remains possible that *P. psidii* infections of native eucalypts in remote areas of Australasia have gone undetected to date. If sites exist where eucalypts and *P. psidii* have co-evolved, cryptic rust disease could easily go undetected until suitable environmental conditions and susceptible germplasm were available that allowed significant epidemiological spread. More extensive pathogen surveys in the native range of eucalypts are required to assess the feasibility of this alternative hypothesis. If eucalypt plants infected with *P. psidii* were imported to Brazil several times, this might account for the higher genetic and genotypic diversities of the eucalypt-associated biotype compared with the guava-associated biotype in Brazil. Alternatively, the higher genotypic diversity and larger number of MLGs observed in the eucalypt-associated population compared with the guava-associated population may have resulted from mutations occurring over short timescales in large population sizes of the pathogen associated with widespread commercial cultivation of eucalypts. Supporting this latter hypothesis, most of the connections among eucalypt-associated MLGs in the network corresponded to single mutational events that could have occurred recently, driven by large eucalypt-associated populations of *P. psidii*.

The Myrtaceae is an extremely large family of woody plants with at least 5650 species in 130–150 genera (Govaerts *et al.* 2008) that are widely distributed in tropical and subtropical regions primarily in the southern hemisphere. Thus, a myriad of potential opportunities exist for the evolution of a rust pathogen capable of infecting eucalypts. Because the eucalypt biotype appears to be specifically associated with eucalypts in Brazil, it might be speculated that this biotype evolved on other myrtaceous relatives of eucalypts, such as species within the genera *Arillastrum* (endemic to New Caledonia), *Corymbia* (native to Australasia/Oceania), or *Angophora* (native to Australasia/Oceania; Wilson *et al.* 2001; Ladiges *et al.* 2003; Biffin *et al.* 2010). However, other evolutionary processes must also be considered when evaluating potential sources of the eucalypt-associated biotype. In this context, it should be considered that eucalypt ancestors existed in South America during the early Eocene (ca. 52 mya) indicating that eucalypt evolution was not restricted to Australasia (Gandolfo *et al.* 2011). Thus, it remains plausible that the eucalypt-associated pathogen could have originated via long-term, co-evolutionary processes with one or more myrtaceous species native to South America or Central America. Additional surveys of *P. psidii* on native myrtaceous species in South and Central America are needed to better assess the evolution of this rust pathogen in these regions.

The demonstrated existence of *P. psidii* biotypes profoundly changes the commonly held view that invasive pathogen risks can be assessed at the species level. The recent introduction of *P. psidii* to Hawaii, Australia and South Africa demonstrate the invasive capacity of a single MLG (Loope 2010; Carnegie & Cooper 2011; Zhong *et al.* 2011; Loope & Uchida 2012; Roux *et al.* 2013) that appears to have a wide host range (Loope 2010; Carnegie & Lidbetter 2012). However, the existence of host-associated biotypes in Brazil raises the possibility that introductions of additional biotypes of *P. psidii* could cause further economic and ecological damage to native and planted Myrtaceae in diverse global regions. The genetic diversity, host range and environmental requirements of *P. psidii* biotypes must be determined to better assess invasive risks to diverse myrtaceous species in widely ranging geographic regions worldwide. Because different *P. psidii* biotypes may display different ecological behaviour and represent distinct invasive threats, continued studies of *P. psidii* populations from diverse geographic locations are needed to monitor the spread of this pathogen, better assess the invasive risks posed by different biotypes and search for the evolutionary origin of invasive biotypes.

Conclusions

Host species strongly influences the population structure of *P. psidii* in Brazil and provides evidence for host-associated genotypes of the pathogen and demonstrates the existence of distinct host-adapted biotypes of *P. psidii*. The hypothesis that a eucalypt-infecting biotype of *P. psidii* in Brazil originated from a guava-infecting biotype is strongly rejected by all our analyses, but the source of the eucalypt-associated biotype cannot be determined from available data due to limited sampling of *P. psidii* from other myrtaceous hosts. Host-associated biotypes of *P. psidii* represent distinct invasive threats to diverse myrtaceous species distributed worldwide and must be considered in assessments of invasive risk. The existence of host-associated biotypes may also indicate that *P. psidii* is undergoing (or has undergone) speciation driven by host, eventually resulting in cryptic species

within a so called '*P. psidii* complex'. This hypothesis is currently being tested using phylogenetic and phylogeographic approaches, as a joint project of the Universidade Federal de Viçosa, USDA-Forest Service, Washington State University, University of Hawaii at Manoa, and University of Tasmania. Our data provide a baseline for ongoing studies of genetic variation of other globally distributed populations of *P. psidii*, for surveillance of potential pathways of *P. psidii* introduction into new regions and to evaluate invasive threats of different *P. psidii* biotypes in diverse global regions where myrtaceous species are grown.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Plot of mean ln likelihoods of the data assuming $K = 1$ to $K = 7$ populations estimated using the Bayesian clustering algorithm implemented in STRUCTURE v2.3.4. Error bars represent standard deviations for likelihoods from 10 runs of the MCMC sampler for each K.

Table S1. Host and geographic origin of *Puccinia psidii* samples.

Table S2. Microsatellite locus primers used to amplify ten microsatellite loci from *Puccinia psidii* sampled from seven myrtaceous hosts in Brazil and eucalypt in Uruguay.

Table S3. Probabilities of repeated sampling of multilocus genotypes arising from sexual reproduction in the eucalypt-associated and guava-associated populations of *Puccinia psidii* in Brazil.

Table S4. Confidence in scenario choice for emergence of *Puccinia psidii* on eucalypts in Brazil.