Genetic diversity of the myrtle rust pathogen (Austropuccinia psidii) in the Americas and Hawaii: Global implications for invasive threat assessments

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Summary

Since the myrtle rust pathogen (*Austropuccinia psidii*) was first reported (as *Puccinia psidii*) in Brazil on guava (*Psidium guajava*) in 1884, it has been found infecting diverse myrtaceous species. Because *A*. *psidii* has recently spread rapidly worldwide with an extensive host range, genetic and genotypic diversities were evaluated within and among *A*. *psidii* populations in its putative native range and other areas of myrtle rust emergence in the Americas and Hawaii. Microsatellite markers revealed several unique multilocus genotypes (MLGs), which grouped isolates into nine distinct genetic clusters [C1–C9 comprising C1: from diverse hosts from Costa Rica, Jamaica, Mexico, Puerto Rico, and USA-Hawaii, and USA-California; C2: from eucalypts (*Eucalyptus* spp.) in Brazil/Uruguay and rose apple (*Syzygium jambos*) in Brazil; C3: from eucalypts in Brazil; C4: from diverse hosts in USA-Florida; C5: from Java plum (*Syzygium cumini*) in Brazil; C6: from guava and Brazilian guava (*Psidium guineense*) in Brazil; C7: from pitanga (*Eugenia uniflora*) in Brazil; C8: from allspice (*Pimenta dioica*) in Jamaica and sweet flower (*Myrrhinium atropurpureum*) in Uruguay; C9: from jabuticaba (*Myrciaria cauliflora*) in Brazil]. The C1 cluster, which included a single MLG infecting diverse host in many geographic regions, and the closely related C4 cluster are considered as a “Pandemic biotype,” associated with myrtle rust emergence in Central America, the Caribbean, USA-Florida, USA-Hawaii, Australia, China-Hainan, New Caledonia, Indonesia and Colombia. Based on 19 bioclimatic variables and documented occurrences of *A*. *psidii* contrasted with reduced sets of specific genetic clusters (subnetworks, considered as biotypes), maximum entropy bioclimatic modelling was used to predict geographic locations with suitable climate for *A. psidii* which are at risk from invasion. The genetic diversity of *A*. *psidii* throughout the Americas and Hawaii demonstrates the importance of recognizing biotypes when assessing the invasive threats posed by *A*. *psidii* around the globe.

1 INTRODUCTION

Biological invasions of pathogens are impacting many forest ecosystems around the globe, with losses in biodiversity and associated impacts on ecosystem health. The enormous social, economic and ecological costs associated with invasions by forest pathogens and emerging diseases have been previously assessed (Pimentel, 2011), and several examples of novel infectious agent-caused species decline, extirpation or extinction have been documented (Martel et al., 2013; Meeus, Brown, De Graaf, & Smagghe, 2011). The movement of pathogens has increased due to the importation of wood products and live plant trade (Roy et al., 2014; Santini et al., 2013; Stenlid, Oliva, Boberg, & Hopkins, 2011; Wingfield, Slippers, Roux, & Wingfield, 2001). As obligate pathogens, rust fungi are likely moved via live plant materials, and their pathways of entry can elude the detection of infected plant materials, especially those with latent stages of infection (Rossman, 2009). Forest rust pathogens, such as *Cronartium ribicola* (cause of white pine blister rust) and *Melampsora larici-populina* (cause of Eurasian

poplar leaf rust), spread to new geographic regions and pose substantial threats. Autoecious rust pathogens have the ability to produce infectious urediniospores on one host, which appears to be an adaptive trait that allows extensive spread via copious spores, possibly driving adaptation towards new geographical areas and/or new hosts (Ali et al., 2014; Helfer, 2014; Morin, Aveyard, Lidbetter, & Wilson, 2012). Prevention of introduction and detection/eradication before establishment occurs is arguably the best measure for reducing damage from invasive rust pathogens (Roy et al., 2014; Santini et al., 2013).

Myrtle rust, also known as guava rust or eucalypt rust, caused by *Austropuccinia psidii* (G. Winter) Beenken (Basidiomycota, Pucciniales), which was previously known as *Puccinia psidii* (Beenken, 2017), is a primary example of an emerging forest disease caused by an autoecious rust pathogen. *Austropuccinia psidii* is a biotrophic fungus that was first reported (as *P*. *psidii*) in Brazil from guava in 1884 (*Psidium guajava*; Winter, 1884) and later from non-native eucalypt (*Corymbia citriodora*, syn. *Eucalyptus citriodora*; Joffily, 1944). Considered to be of neotropical origin, the pathogen has also been reported to infect other diverse myrtaceous hosts in South America (e.g., Granados et al., 2017; Kern & Toro, 1935; Mohali & Aime, 2016; Pérez, Wingfield, Altier, Simeto, & Blanchette, 2011; Simpson, Thomas, & Grgurinovic, 2006; Tommerup, Alfenas, & Old, 2003), the Caribbean (MacLachlan, 1938), California, USA (Mellano, 2006; Zambino & Nolan, 2011), Florida, USA (Marlatt & Kimbrough, 1979), Hawaii, USA (Uchida, Zhong, & Kilgore, 2006), Japan (Kawanishi et al., 2009), Australia (Carnegie et al., 2010), Hainan, China (Zhuang & Wei, 2011), and most recently South Africa (Roux, Greyling, Coutinho, Verleur, & Wingfield, 2013), New Caledonia (Giblin, 2013), Indonesia (McTaggart et al., 2016) and Singapore (de Plessis et al., 2017). In addition to rapid global spread of the myrtle rust pathogen in recent years, emergent natural epiphytotics (e.g., Rayachhetry, Elliott, & Van, 1997; Rayamajhi, Pratt, Klopfenstein, Ross-Davis, & Rodgers, 2013; Uchida & Loope, 2009) and new host reports (e.g., Pérez et al., 2011; Rodas et al., 2015; Telechea, Rolfo, Coutinho, & Wingfield, 2003; Zambino & Nolan, 2011) continue to be observed and documented within the Americas and Hawaii.

Austropuccinia psidii has a wide host range, with more than 450 known host species within the Myrtaceae (Giblin & Carnegie, 2014), a large plant family of more than 5,600 species (Grattapaglia et al., 2012). This biotrophic rust pathogen is hypothesized to be autoecious (Morin, Talbot, & Glen, 2014), completing its life cycle on actively growing shoots and fruits of susceptible hosts under conditions of moderate temperatures and extended periods of high humidity (Coutinho, Wingfield, Alfenas, & Crous, 1998; Glen, Alfenas, Zauza, Wingfield, & Mohammed, 2007). In nature, urediniospores are commonly observed, with teliospores and basidiospores comparatively less frequent, and spermagonial and aecial stages have never been observed (Glen et al., 2007). The disease kills shoot tips, causing a loss of apical dominance, and can ultimately result in mortality (Uchida & Loope, 2009). This pathogen poses a large risk to the nearly 20 million hectares of eucalypts currently planted around the globe (e.g., Coutinho et al., 1998; Glen et al., 2007; Tommerup et al., 2003) and is a threat to native biodiversity (Carnegie et al., 2016; Pegg et al., 2014). Susceptible species are dominant components of flora in widespread global areas, such as Oceania Australia, Southeast Asia, South and Central America and southern Africa; thus, disease outbreaks could severely alter the structure, composition and function of these ecosystems.

Because of the urgent need to better understand the invasive threat posed by *A*. *psidii*, several previous studies have used bioclimatic modelling to predict the potential geographic range of this pathogen (e.g., Alvares et al., 2017; Booth & Jovanovic, 2012; Booth, Old, & Jovanovic, 2000; Elith, Simpson, Hirsch, & Burgman, 2013; Glen et al., 2007; Kriticos & Leriche, 2008; Kriticos, Morin, Leriche, Anderson, & Caley, 2013; Magarey et al., 2007; Ramsfield, Dick, Bulman, & Ganley, 2010; Roux et al., 2015). Most of these previous risk assessments for *A*. *psidii* are directed at specific geographic regions and/or did not consider distinct genetic groups within *A*. *psidii* that may have different ecological behaviours or climatic requirements. As pointed out by Elith et al. (2013), for predicting areas at risk from *A. psidii* invasion, it is critical to determine how different taxonomic or genetic groups of *A. psidii* may behave under climatic conditions associated with each geographic area.

Understanding the genetic diversity of *A*. *psidii* is critical for understanding host associations, potential pathways of spread and climatic requirements, all of which facilitate the development of management strategies. In previous work, 10 microsatellite loci were successfully used to characterize *A*. *psidii* populations associated with different hosts in multiple regions of Brazil (Graça et al., 2013). Building on that previous work, this study sought to assess genetic and genotypic diversities within *A*. *psidii* collections across a wider geographic range, including its putative native range as well as areas of emergence in the Americas and Hawaii. Collection data for *A*. *psidii* and associated genetic analyses also were applied in preliminary bioclimatic modelling to demonstrate the utility of genetic analysis in predicting threats posed by *A*. *psidii* and its genetic groups.

2 MATERIALS AND METHODS

2.1 Sampling

A total of 226 single-pustule isolates of *A. psidii* were genotyped in this study. Of these, 147 isolates collected from seven taxa of Myrtaceae in Brazil and Uruguay are reported in Graça et al. (2013). An additional 79 isolates of *A. psidii* collected from 15 different host species (11 genera) from six countries between 2008 and 2013 were also genotyped and are included in this study (Table 1), which include 50 isolates previously collected from Hawaii, USA (Graça, 2011). Accordingly, this study includes 29 novel isolates, representing nine host taxa and six countries. Uredinial pustules of *A. psidii* were excised from infected host tissue and stored in 1.5-ml microcentrifuge tubes with filter paper secured over an open top within a 50-ml conical tube containing approximately 20 g dry silica gel.

TABLE 1 Geographic origin, host and genetic cluster of Austropuccinia psidii samples

Origin	Host	*N*	BAPS cluster[3]
Brazil	*Eucalyptus* spp.	70	C2 and C3
	Eugenia uniflora	2	C7
	Myrciaria cauliflora	3	C9
	Psidium guajava	63	C6
	Psidium guineense	2	C6
	Syzygium cumini	4	C5
	Syzygiumjambos	3	C2
Costa Rica	*Callistemon lanceolatus*	2	C1
Jamaica	*Pimenta dioica*	6	C8
	Syzygiumjambos	4	C1
Mexico	*Syzygiumjambos*	1	C1
Puerto Rico	*Syzygium jambos*	1	C1
Uruguay	*Eucalyptus grandis*	1	C2
	Eucalyptus globulus	3	C2
	Myrrhinium atropurpureum	1	C8
USA—Florida	*Melaleuca quinquenervia*	5	C4
	Myrcianthes fragrans	1	C4
	Rhodomyrtus tomentosa	2	C4
	Syzygium jambos	2	C4
USA - Hawaii	*Eugenia koolauensis*	3	C1
	Melaleuca quinquenervia	4	C1
	Metrosideros excelsa	1	C1
	Metrosideros polymorpha	9	C1
	Myrtus communis	1	C1
	Rhodomyrtus tomentosa	2	C1
	Syzygium cumini	1	C1
	Syzygium jambos	28	C1
	Syzygium malaccense	1	C1

[a]Bayesian analysis of population structure (BAPS) identified nine genetic clusters (C1-C9) among 226 *Austropuccinia psidii* isolates.

2.2 DNA isolation and genotyping

For the original 147 isolates included in Graça et al. (2013), genomic DNA was extracted directly from each pustule (containing fungal and host tissue) using a modified CTAB-based protocol (Doyle & Doyle, 1987). For all other isolates, genomic DNA was extracted from dried pustules using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's recommendations. Six of the 10

TABLE 2 Six microsatellite loci amplified among 226 *Austropuccinia psidii* isolates

Locus	GenBank accession	Primer sequence (5'^3')	GC content (%)	Number of alleles
PpSSR012	EF523501	F: TTCAATCCCCATAAGGCTTTC	43	6
		R: AAATCCTGAGTCTTCTTCCCC	48	
PpSSR014	EF523502	F: TTCGACATCCAACGCTCTCAT	48	5
		R: AAAGGCTAAGTGAATGGGCA	45	
PpSSR018	EF523503	F: AGCCTTCTCTCTCCTCCGTTA	52	4
		R: TCAGGAAGGACAAGACCAAGT	48	
PpSSR022	EF523504[a]	F: CCTTTAGGCTGTGGTTTCCA	50	8
		R: GCCCACTCTGTCAAGAGGAA	55	
PpSSR087	EF523507[a]	F: AAGAACGTGAACGGGAATGA	45	10
		R: GAAATGCCAGACGAAGGGTA	50	
PpSSR102	EF523508[a]	F: TGACTTTAATCATCTTCAAAACCAA	28	9
		R: ACCAATCCCCTTCCTTCATC	50	

[a]New primers designed from those deposited in GenBank (Zhong, Yang, & Alfenas, 2008) using Primer3 (Rozen & Skaletsky, 2000).

microsatellite loci used in Graça et al. (2013) were scored for *A. psidii* isolates derived from diverse hosts and geographic locations; the remaining four loci amplified inconsistently and were not included in the analysis (Table 2). PCR amplifications were performed using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation period of 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 plate, and scoring was repeated as necessary to estimate an error rate for genotyping accuracy. 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).

Isolates were assigned to genetic clusters using a Bayesian genetic clustering algorithm implemented in BAPS v5.3 (Corander & Marttinen, 2006). BAPS analyses were performed with the complete data set ($N = 226$). Cluster analyses were performed at the individual population level. Posterior probabilities were estimated for inferred partitions ($K = 1$ to $K = 12$) assuming an admixture model. Admixture within populations was also tested using admixture with mixed clustering, a minimum population size of 2, 50 iterations and two reference individuals from each population with 10 iterations for reference individuals. Population genetic analyses of all samples grouped by genetic cluster were performed using GenAlEx v6.501. The following genetic parameters were determined the following: number of different alleles (N_A), number of effective alleles (N_E), number of private alleles (N_P), Shannon's information index (I), observed heterozygosity (H_O), expected heterozygosity (H_E), unbiased expected heterozygosity ($_UH_E$) and fixation index (F). In addition, Nei's unbiased genetic distance and pairwise F_{ST} for all populations were also estimated, including estimates of heterozygosity within populations and in pairwise comparisons. In some cases, sample sizes of populations were too small to make accurate estimations (F_{ST}); Populations C7 and C9 were not included in F_{ST} estimations because sample sizes were below four (Peakall & Smouse, 2006).

Principal coordinates analysis (PCoA), based on a covariance matrix with data standardization (Smouse & Peakall, 1999), was performed using the complete data set (*N* = 226). To examine relationships among genotypes, a minimum-spanning network (MSN) was estimated in Poppr (Kamvar, Tabima, & Grunwald, 2014) using the genetic distance measure of Bruvo, Michiels, D'Souza, and Schulenburg (2004).

2.3 Bioclimatic modelling

MaxEnt (Maximum Entropy Species Distribution Modeling) v3.3.3K models were run based on 1) all documented *A. psidii* occurrences in our data set (*N* = 403) and 2) reduced sets of three genetic subnetworks (see Results) including, biotypes C2/C3 [isolates from eucalypts (*Eucalyptus* spp.) in Brazil/Uruguay and rose apple (*S. jambos*) in Brazil; *N* = 80], C1/C4 [occurrence points from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida, combined with a set of 81 occurrence locations from Australia that are considered to be C1 genotype (Machado, Alfenas, Alfenas, Mohammed, & Glen, 2015; Sandhu, Karaoglu, Zhang, & Park, 2016); *N* = 137] and C6 [isolates from guava (*P. guajava*) and Brazilian guava (*P. guineense*) in Brazil; *N* = 60], coupled with 19 bioclimatic variables derived from the WorldClim database (worldclim.org) (Table 4). MaxEnt was applied because it performs well with limited presence-only data (Elith et al., 2006, 2011). Random subsampling using a 25% test percentage was used to generate statistical results among 15 replicate runs. Quantum GIS (QGIS; http://www.qgis.org/en/site/) was used to create the final outputs using MaxEnt's cumulative output. Each cumulative value is the sum of probabilities of cells less than or equal to the cell grid, times 100. The permutation importance was generated from MaxEnt to determine the importance of input variables (19 bioclimatic variables in this study) (Table 4). The importance is measured based on the final model only, which is determined by randomly permuting the values of that variable among the training presence and background data and measuring the resulting drop in area under curve (AUC, which reflects the ability to separate known presences from background points) (Phillips, Anderson, & Schapire, 2006).

3 RESULTS

Twenty-three unique multilocus genotypes (MLGs) were identified among *A. psidii* isolates collected from Brazil, Costa Rica, Jamaica, Mexico, Puerto Rico, Uruguay and USA using six microsatellite loci. Results of the BAPS analyses indicated that *K* = 9 was the best estimate of the number of genetic clusters (estimated *ln* probability of data = −1782.56; Figure 1), assuming no recombination. The following nine genetic clusters were identified as follows: (C1) isolates from diverse hosts from Costa Rica, Jamaica, Mexico, Puerto Rico and USA-Hawaii, (C2) isolates from eucalypts in Brazil/Uruguay and rose apple in Brazil, (C3) isolates from eucalypts in Brazil, (C4) isolates from diverse hosts in Florida, USA, (C5) isolates from Java plum (*S. cumini*) in Brazil, (C6) isolates from guava and Brazilian guava in Brazil, (C7) isolates from pitanga (*Eugenia uniflora*) in Brazil, (C8) isolates from allspice (*Pimenta dioica*) in Jamaica and sweet flower (*Myrrhinium atropurpureum*) in Uruguay and (C9) isolates from jabuticaba (*Myrciaria cauliflora*) in Brazil.

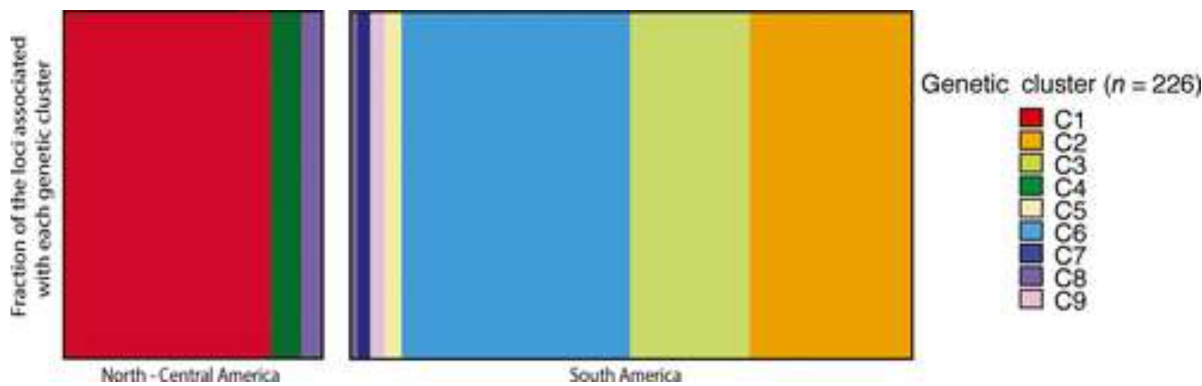

Figure 1 Population structure of 226 *Austropuccinia psidii* samples inferred using a Bayesian clustering algorithm implemented in BAPS with each individual represented by a vertical line partitioned into shaded segments corresponding to the isolate's estimated mean membership coefficient for K = 9 genetic clusters; mean $Ln\mathrm{P}(K) = -1782.56$. Genetic clusters (C1–C9) are described in Table 1

All loci were polymorphic, with four to 10 alleles detected per locus (Table 3). Number of different alleles per locus (N_A) ranged from 1.33 for C6 (65 isolates from guava and Brazilian guava in Brazil) to 3.00 for C4 (10 isolates from diverse hosts in Florida, USA) and the effective number of alleles per locus (N_E) ranged from 1.33 for C6 to 2.38 for C4. The most diverse populations were C1 (58 isolates from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico and USA-Hawaii) and C4 (10 isolates from diverse hosts in Florida, USA) with two and three private alleles (N_P), respectively, and observed heterozygosities (H_O) of 1.00. The least diverse population was C6 (65 isolates from guava and Brazilian guava in Brazil) with an N_P of 0 and H_O of 0.33. All clusters had negative fixation indices (F ranged from −0.76 to −1.00) indicating an excess of heterozygotes.

TABLE 3 Summary of genetic and genotypic diversities among clusters[3] of *Austropuccinia psidii*

	C1	C2	C3	C4	C5	C6	C7	C8	C9
N	58	44	33	10	4	65	2	7	3
MLG	4	4	1	5	1	1	1	5	1
^{N}A	2.50 (0.50)	2.00 (0.37)	1.83 (0.17)	3.00 (0.37)	-	1.33 (0.21)	-	-	-
^{N}E	2.02 (0.02)	1.75 (0.25)	1.83 (0.17)	2.38 (0.15)	-	1.33 (0.21)	-	-	-
^{N}P	2	1	0	3	-	0	-	-	-
I	0.72 (0.03)	0.53 (0.17)	0.58 (0.12)	0.93 (0.08)	-	0.23 (0.15)	-	-	-
^{H}O	1.00 (0.00)	0.67 (0.21)	0.83 (0.17)	1.00 (0.00)	-	0.33 (0.21)	-	-	-
^{H}e	0.51 (0.01)	0.35 (0.11)	0.42 (0.08)	0.57 (0.03)	-	0.17 (0.11)	-	-	-
U^{H}E	0.51 (0.01)	0.36 (0.11)	0.42 (0.08)	0.60 (0.03)	-	0.17 (0.11)	-	-	-
F	-0.98 (0.02)	-0.90 (0.05)	-1.00 (0.00)	-0.76 (0.08)	-	-1.00 (0.00)	-	-	-

N, number of samples; MLG, number of multilocus genotypes; N_A, number of different alleles; N_E, number of effective alleles; N_P, number of private alleles; I, Shannon's information index; H_O, observed heterozygosity; H_E, expected heterozygosity; $_UH_E$, unbiased expected heterozygosity; F, fixation index; -, not estimated due to limited sample size.
[a]Clusters are described in Table 1.

To assess genetic relationships among clusters, the average F_{ST} for pairwise comparisons were calculated. The overall cluster differentiation was F_{ST} = 0.471. Pairwise F_{ST} values ranged from 0.46

between C5 (four isolates collected from Brazil on *S. cumini*) and C6 (65 isolates from guava and Brazilian guava in Brazil) to 0.06 between C1 (58 isolates from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico and USA-Hawaii) and C4 (10 isolates from diverse hosts in Florida, USA). C2 (44 isolates from eucalypts in Brazil and Uruguay and from rose apple in Brazil) was genetically distinct from C5 (four isolates collected from Brazil on *S. cumini*) and C6 (65 isolates from guava and Brazilian guava in Brazil) with pairwise F_{ST} = 0.40 and 0.39, respectively.

The first two axes of the PCoA explained 79% of total variation (Figure 2). The first axis explained 43% of the variation and separated C6 (isolates from guava and Brazilian guava in Brazil, which grouped low on coordinate 1) from the other clusters (which grouped intermediate to high on coordinate 1). The second axis, which explained an additional 36% of the variation, separated C2 (isolates from eucalypts in Brazil/Uruguay and rose apple in Brazil), C3 (isolates from eucalypts in Brazil), C7 (isolates from pitanga in Brazil), and C9 (isolates from jabuticaba in Brazil) from C8 (isolates from allspice in Jamaica and sweet flower in Uruguay), C5 (isolates from Java plum in Brazil), C4 (isolates from diverse hosts in Florida, USA), and C1 (isolates from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, and USA-Hawaii).

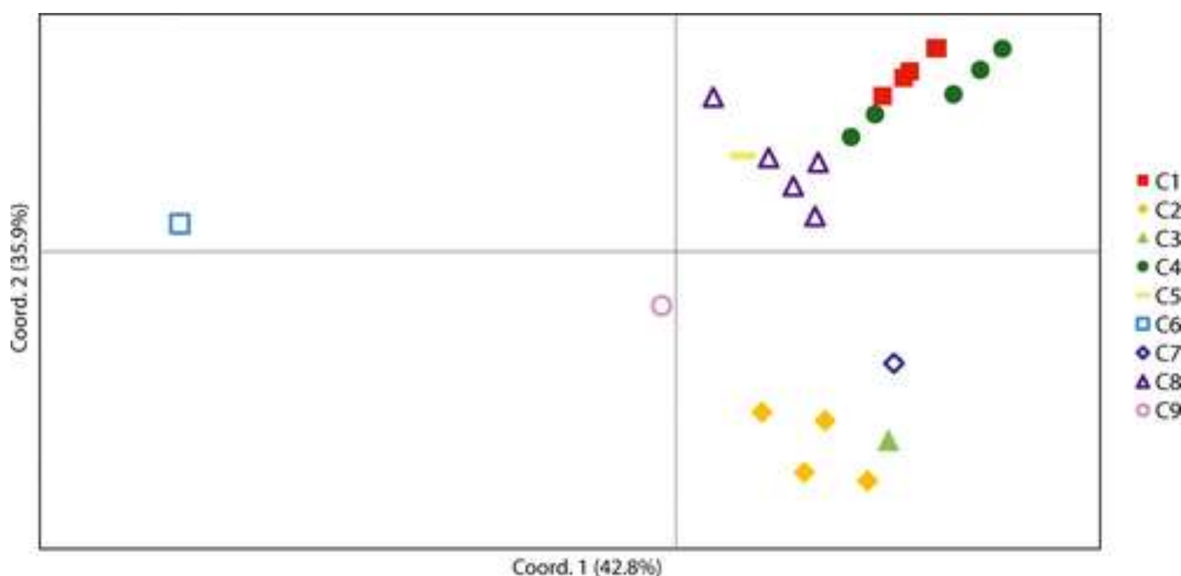

Figure 2 Principal coordinates analysis of the 23 mutlilocus genotypes of 226 *Austropuccinia psidii* isolates among nine clusters (C1–C9) as identified by BAPS (see Figure 1 and Table 1) based on a covariance matrix with data standardization. The first two axes explain 79% of the observed variation. Genetic clusters (C1–C9) are described in Table 1

The minimum-spanning network supported the BAPS and PCoA analyses, revealing distinct subnetworks associated with each of the genetic clusters described above (Figure 3). Each subnetwork was separated by large genetic distances (Bruvo distance >0.20) and represented allelic variation across multiple loci. The first major subnetwork comprising C1 (isolates from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico and USA-Hawaii) and C4 (isolates from diverse hosts in Florida, USA) was separated from a subnetwork comprising C5 (a single MLG associated with isolates from Java plum in Brazil) by a distance of 0.58 and from another major subnetwork comprising C8 (isolates from allspice in Jamaica and sweet flower in Uruguay) by a Bruvo distance of 0.33. Most connections between genotypes in this C1/C4 subnetwork represented single mutational steps (equivalent to a distance of

approximately 0.04). A third major subnetwork, which comprised C2 (isolates from eucalypts in Brazil/Uruguay and rose apple in Brazil) and C3 (isolates from eucalypts in Brazil), was separated from C9 (single MLG of isolates from jabuticaba in Brazil) by a Bruvo distance of 0.51, and from the subnetwork comprising C8 (isolates from allspice in Jamaica and sweet flower in Uruguay) by a distance of 0.22. The final major subnetwork comprised C6 (single MLG associated with isolates from guava and Brazilian guava in Brazil) and was separated from C7 (isolates from pitanga in Brazil) by a distance of 0.25 and from C8 (isolate originating from sweet flower in Uruguay) by a distance of 0.25.

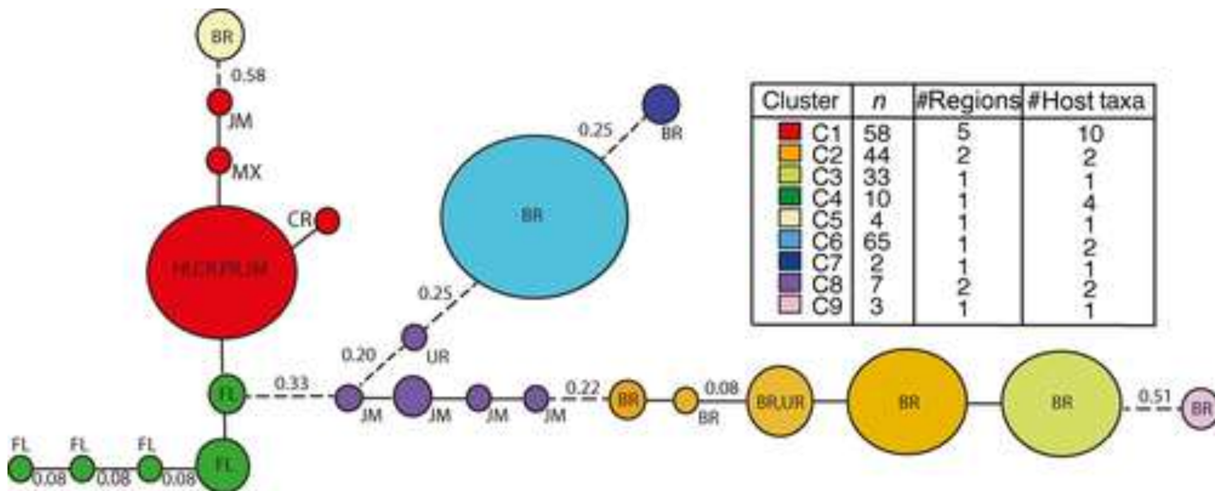

Figure 3 Minimum-spanning network of *Austropuccinia psidii* microsatellite multilocus genotypes (MLGs) sampled from Brazil (BR), Costa Rica (CR), Jamaica (JM), Mexico (MX), Puerto Rico (PR), Uruguay (UR), and Florida (FL) USA, and Hawaii (HI) USA on 18 hosts. MLGs are represented by BAPS genetic clusters: C1 represents MLGs from Costa Rica on crimson bottlebrush (*Callistemon lanceolatus*), Jamaica, Mexico, Puerto Rico on rose apple (*Syzygium jambos*) and Hawaii, USA on koʻolau eugenia (*Eugenia koolauensis*), broad-leaved paperbark (*Melaleuca quinquenervia*), pōhutukawa (*Metrosideros excelsa*), ʻōhiʻa lehua (*M. polymorpha*), common myrtle (*Myrtus communis*), rose myrtle (*Rhodomyrtus tomentosa*), Java plum (*S. cumini*), rose apple and Malay rose apple (*S. malaccense*); C2 represents MLGs collected from Brazil on eucalypts (*Eucalyptus* spp.) and rose apple and from Uruguay on eucalypts (*Eucalyptus grandis* and *E. globulus*); C3 represents one MLG collected from Brazil on eucalypts; C4 represents MLGs collected from Florida, USA on broad-leaved paperbark, twin berry (*Myrcianthes fragrans*), rose myrtle and rose apple); C5 represents one MLG collected in Brazil on Java plum; C6 represents one MLG collected in Brazil on guava (*Psidium guajava*) and Brazilian guava (*P. guineense*); C7 represents one MLG collected in Brazil on pitanga (*Eugenia uniflora*); C8 represents MLGs collected from Jamaica on allspice (*Pimenta dioica*) and Uruguay on sweet flower (*Myrrhinium atropurpureum*); C9 represents one MLG collected from Brazil on jabuticaba (*Myrciaria cauliflora*). Sizes of circles are proportional to MLG frequency. 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 are separated by distances >0.20. Loops with dotted lines in the network (i.e., with C1-, C5- and C8-associated MLGs) indicate multiple, tied minimum-spanning trees

The MaxEnt bioclimatic model using all records of *A. psidii* occurrence (*N* = 403) shows a prediction with many areas throughout the world as having some degree of probability of suitable climate for the myrtle rust pathogen (Figure 4). Models based on reduced sets of C1/C4 (137 occurrence locations from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida and Australia), C2/C3 (80 occurrence locations from eucalypts in Brazil/Uruguay and rose apple in Brazil) and C6 (60 occurrence locations from guava and Brazilian guava in Brazil) shows some differences in the predictions of suitable climate space (Figures 4-6). For the all-inclusive model, the major contributors (i.e., >10%) to

permutation importance were isothermality, maximum temperature of warmest month and precipitation of the driest month (Table 4). In addition, isothermality and maximum temperature of warmest month were also major contributors in the C1/C4 and C2/C3 models. Precipitation in the coldest quarter and mean temperature of the wettest quarter were also major contributors in the C1/C4 model; whereas, temperature seasonality and precipitation of the driest month were major contributors in the C2/C3 model. In contrast, temperature seasonality and mean temperature of the coldest quarter were major contributors in the C6 model. Precipitation during the driest month, which was one of the largest contributors in the all-inclusive and C2/C3 models, contributed only 0.7% and 6.3% in the C1/C4 and C6 models, respectively. For the C2/C3 and C6 models, temperature seasonality was one of the largest contributors; whereas, it contributed only 3.3% and 7.5% in the all-inclusive and C1/C4 models, respectively.

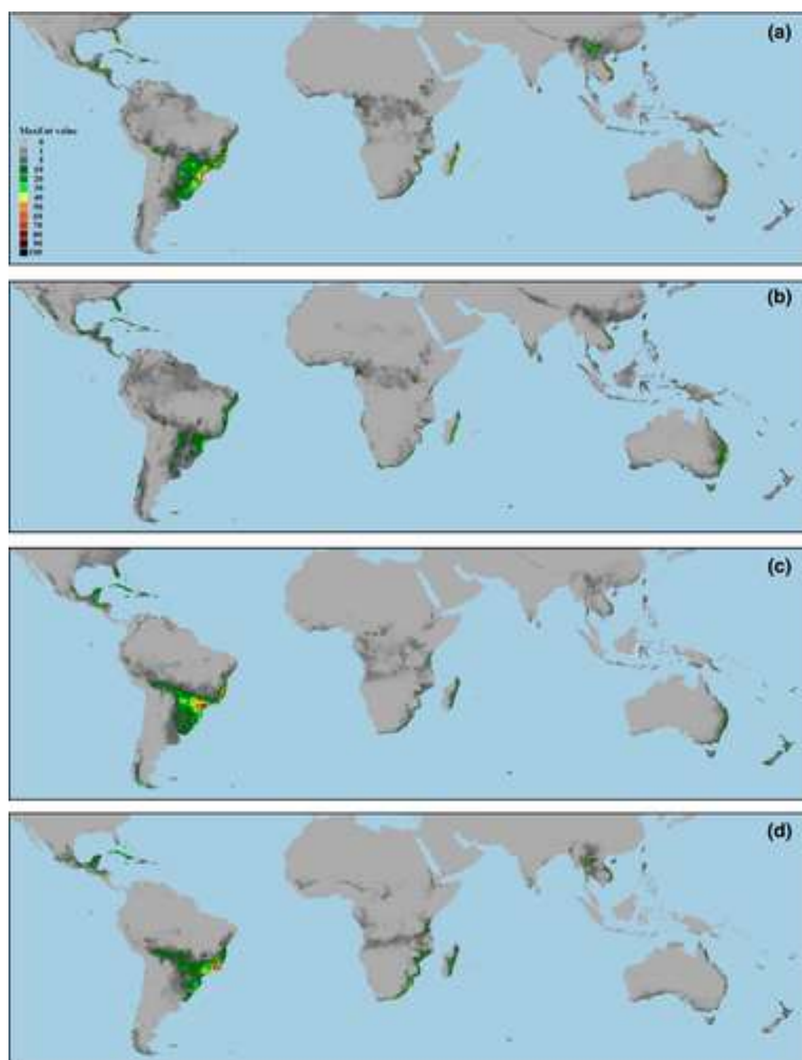

Figure 4 MaxEnt models of suitable climate space (potential distribution) for *Austropuccinia psidii* based on 19 global bioclimatic variables derived from the WorldClim (worldclim.org) database for (a) all genotypes (*N* = 403), (b) C1/C4 biotype (Pandemic: occurrence points from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida and Australia; *N* = 137), (c) C2/C3 biotype (eucalypt/rose apple–Brazil/Uruguay; *N* = 80) and (d) C6 biotype (guava/Brazilian guava–Brazil; *N* = 60)

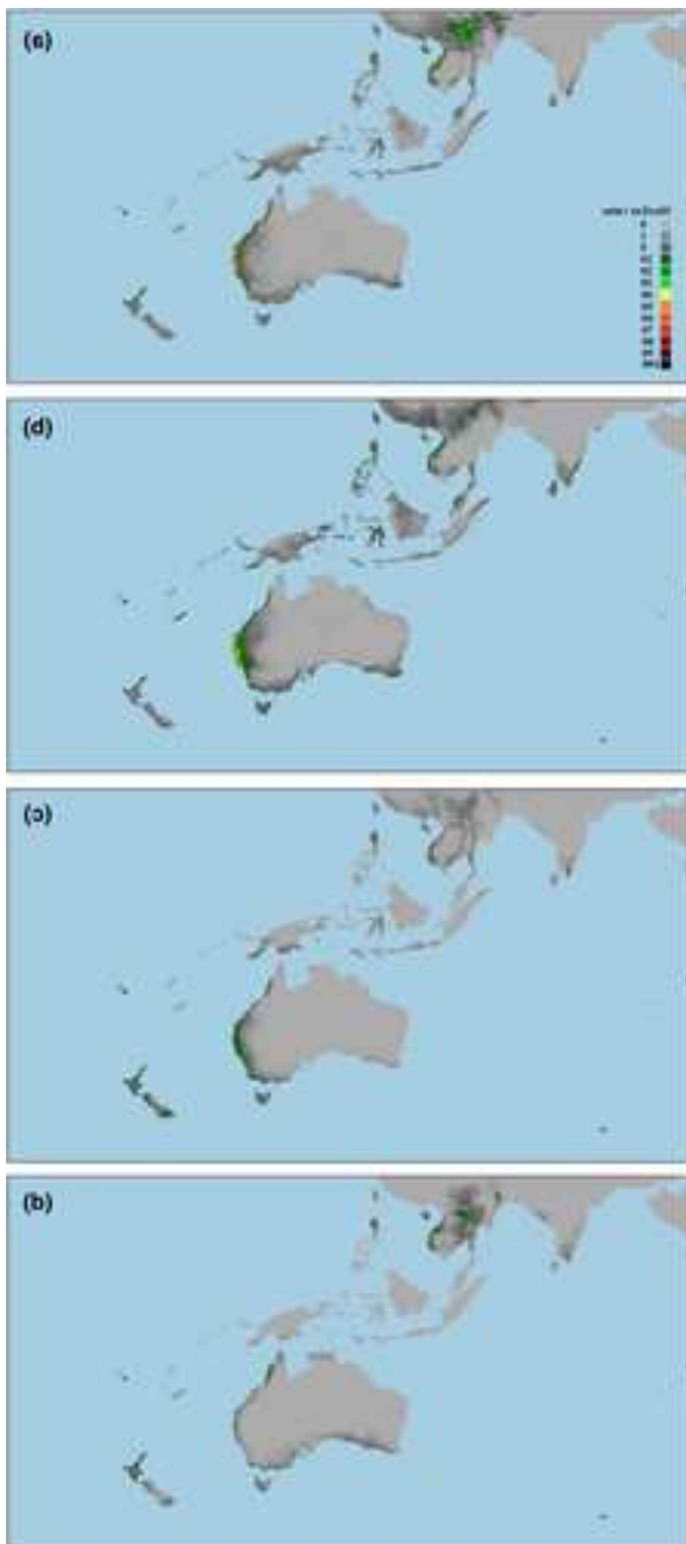

Figure 5 MaxEnt models of suitable climate space (potential distribution) for *Austropuccinia psidii* in Australia, New Zealand, and adjacent regions (based on 19 global bioclimatic variables derived from the WorldClim (worldclim.org) database for (a) all genotypes (*N* = 403), (b) C1/C4 biotype (Pandemic: occurrence points from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida and Australia; *N* = 137), (c) C2/C3 biotype (eucalypt/rose apple–Brazil/Uruguay; *N* = 80) and (d) C6 biotype (guava/Brazilian guava–Brazil; *N* = 60)

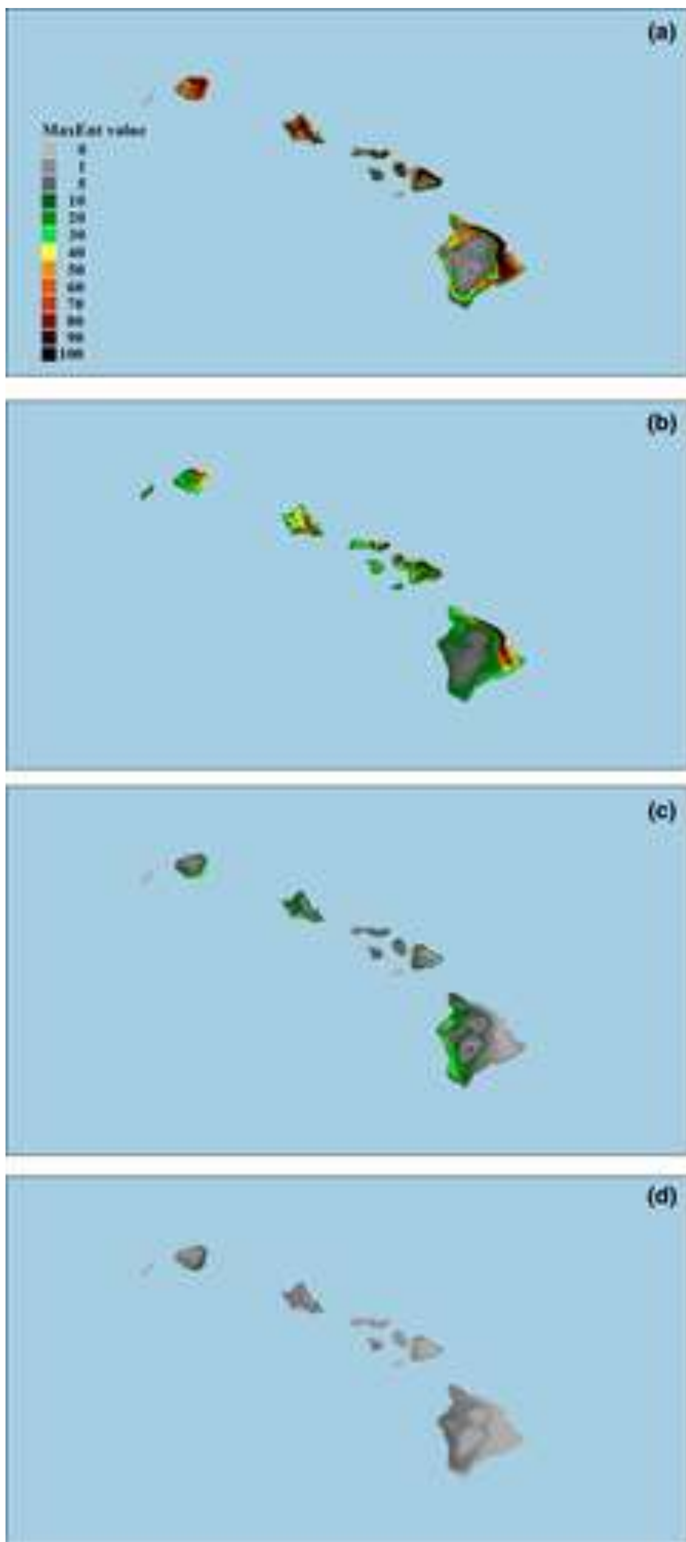

Figure 6 MaxEnt models of suitable climate space (potential distribution) for *Austropuccinia psidii* in Hawaii, USA (based on 19 global bioclimatic variables derived from the WorldClim (worldclim.org) database for (a) occurrence points for all genotypes (*N* = 403), (b) C1/C4 biotype (Pandemic: occurrence points from diverse hosts in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida and Australia; *N* = 137), (c) C2/C3 biotype (eucalypt/rose apple–Brazil/Uruguay; *N* = 80) and (d) C6 biotype (guava/Brazilian guava–Brazil; *N* = 60)

TABLE 4 Summary of Maxent's permutation importance value for each of the 19 bioclimatic variables derived from the WorldClim (worldclim.org) database used to model suitable climate space (potential distribution) based on occurrence points. Variables with the greatest permutation importance (>10%) are in bold. Clusters *Austropuccinia psidii* are described in Table 1

Variable		All inclusive[a]	C1/C4 biotype[b] (Pandemic)	C2/C3 biotype[c] (eucalypt/rose apple-Brazil/Uruguay)	C6 biotype[d] (guava/ Brazilian
Bio1	Annual mean temperature	2.6	0	0.3	0.3
Bio2	Mean diurnal range	2.7	1	0	0
Bio3	Isothermality	33.9	36.6	11.8	0.1
Bio4	Temperature seasonality	3.3	7.5	25.5	41.4
Bio5	Maximum temperature of warmest month	14.9	21.8	12.8	0
Bio6	Minimum temperature of coldest month	0.4	0.1	0	0
Bio7	Temperature annual range	0.5	0.8	0	0
Bio8	Mean temperature of wettest quarter	4.5	12.9	1.5	3.3
Bio9	Mean temperature of driest quarter	0.3	1.8	1.7	0
Bio10	Mean temperature of warmest quarter	2.7	0.6	6.3	0
Bio11	Mean temperature of coldest quarter	0.4	0	2.6	41.8
Bio12	Annual precipitation	4.2	0.1	7.6	0.3
Bio13	Precipitation of wettest month	0.3	0	0.4	0.3
Bio14	Precipitation of driest month	23.6	0.7	13.9	6.3
Bio15	Precipitation seasonality	0.9	2	5.7	0.9
Bio16	Precipitation of wettest quarter	2.6	0	0	0
Bio17	Precipitation of driest quarter	0.3	1.6	0.4	0
Bio18	Precipitation of warmest quarter	1.4	0.4	0.8	0.3
Bio19	Precipitation of coldest quarter	0.5	11.9	8.6	4.9

[a]*All* Austropuccinia psidii *genotypes.*

[b]C1/C4 biotype (Pandemic: isolates from diverse hosts occurring in Costa Rica, Jamaica, Mexico, Puerto Rico, USA-Hawaii/Florida and Australia. [c]C2/C3 biotype (eucalypt/rose apple-Brazil/Uruguay). [d]C6 biotype (guava/Brazilian guava-Brazil).

AUC [area under ROC (receiver operating characteristic curve)] values were generated to calculate model performance based on true positive, false positive, true negative and false-negative rates. All four models were well within the excellent categorical range suggested by Swets (1988), which indicates that model performance was far superior than pure chance (Araújo, Pearson, Thuiller, & Erhard, 2005). With 1.0 representing a perfect value and 0.05 representing pure chance, the average test AUC ± standard deviation values based on 15 replicate runs were 0.984 ± 0.002 (all), 0.991 ± 0.002 (C1/C4), 0.994 ± 0.002 (C2/C3) and 0.996 ± 0.001 (C6).

4 DISCUSSION

This research reveals the existence of multiple genetic clusters of *A. psidii* associated with rust diseases of myrtaceous hosts sampled throughout the Americas and Hawaii. This genetic information is important because, as shown in the bioclimatic modelling, these clusters likely have biological differences that may affect their invasiness potential in different geographic regions. Further, our data show that isolates contained in a single genetic cluster of *A*. *psidii*, namely C1, have spread across a large geographic region associated with several different host taxa. The wide host and geographic ranges of MLGs in this cluster contrasts with the host-associated biotypes described from the putative native range of the pathogen by Graça et al. (2013). In the current study, C1 isolates were sampled from USA-Hawaii [koʻolau eugenia (*Eugenia koolauensis*), broad-leaved paperbark (*Melaleuca quinquenervia*), pōhutukawa (*Metrosideros excelsa*), ʻōhiʻa lehua (*Metrosideros polymorpha*), common myrtle (*Myrtus communis*), rose myrtle (*Rhodomyrtus tomentosa*), java plum, rose apple, and Malay rose apple (*Syzygium malaccense*)], Costa Rica [on crimson bottlebrush (*Melaleuca citrina*)], Jamaica (on rose apple), Mexico (on rose apple) and Puerto Rico (on rose apple). Based on our analyses, the widespread distribution, and the diverse host range, C1 [contained in the C1/C4 subnetwork based on close genetic relatedness (F_{ST} = 0.06)] is hereafter referred to as a component of the "Pandemic biotype."

Perhaps even more noteworthy is that a single MLG within C1 was found on diverse hosts in USA-Hawaii (Graça, 2011), Costa Rica, Jamaica and Puerto Rico. Whether this MLG represents a single clone remains to be determined. In Hawaii, all 50 *A*. *psidii* isolates from nine host species belong to the same MLG, which strongly supports the hypothesis that *A*. *psidii* is an introduced, invasive pathogen in Hawaii (Graça, 2011; Uchida et al., 2006). Further, recent research indicates that the MLG found in Hawaii matches that which is rapidly spreading across Australia since the initial discovery of the disease in 2010 (Machado et al., 2015; Sandhu et al., 2016), and this same genotype was also recently found in China-Hainan (Machado et al., 2015; Zhuang & Wei, 2011), New Caledonia (Giblin, 2013; Machado et al., 2015), Indonesia (McTaggart et al., 2016) and Colombia (Granados et al., 2017). These independent studies provide strong evidence that the MLG found in Australia, China-Hainan, New Caledonia, Indonesia and Colombia is a member of C1. Because of its wide host range and wide geographic distribution, C1 is considered a component of the Pandemic biotype that is associated with myrtle rust emergence in North America (including Central America and the Caribbean), USA-Hawaii, Australia, China-Hainan, New Caledonia and Indonesia.

Closely related to C1 were the five MLGs of C4 cluster (isolates from four host taxa from Florida, USA), which are also considered to represent the Pandemic biotype because of the

very close genetic distance between C1 and C4 (F_{ST} = 0.06; Bruvo's distance = 0.04), compared to between C1 and C5 (F_{ST} = 0.26; Bruvo's distance = 0.58) and between C1 and C8 (F_{ST} = 0.22; Bruvo's distance = 0.33). Marlatt and Kimbrough (1979) documented the first range expansion of myrtle rust into the continental USA on allspice in south Florida in 1977. Decades prior, *A. psidii* was detected on allspice in Jamaica (MacLachlan, 1938; Smith, 1935). *Austropuccinia psidii* behaved as an invasive pathogen on allspice in Jamaica; however, infections of rose apple, which was introduced to Jamaica in 1762 (Asprey & Robbins, 1953), were observed in the country for several years prior to the allspice epidemic of 1935 (Dale, 1955). Two distinct strains of the pathogen were originally reported in Jamaica: one capable of infecting allspice and bay rum (*Pimenta racemosa*), which are native to the Caribbean region, and another infecting rose apple and Malay rose apple, which were both introduced to Jamaica. Neither strain was capable of parasitizing guava in inoculation trials (MacLachlan, 1938). In our study, MLGs collected from rose apple in Jamaica clustered in C1 (Pandemic biotype) and differed by multiple mutational steps across multiple loci from MLGs collected from allspice in Jamaica (C8). If the *A. psidii* isolates from rose apple in Jamaica are representative of the original populations observed several years prior to 1935, it appears unlikely that this population underwent a host shift that allowed it to infect allspice, as postulated by Smith (1935).

Whether or not the pathogen migrated from the Caribbean to Florida or vice versa remains unknown. Unlike the *A. psidii* strains from Jamaica, *A. psidii* detected on allspice in Florida readily infected both allspice and rose apple, as reported by Marlatt and Kimbrough (1979); guava inoculation trials were not conducted. Unfortunately, *A*. *psidii* isolates from allspice from locations outside of Jamaica were not available for this study, so inferences about the origin of the Jamaican allspice cluster (C8) remain limited. It is intriguing that the isolate from native sweet flower from Uruguay clustered with C8 (isolates collected from allspice in Jamaica), but the limited genetic data preclude any further inferences.

Of potential note is the genetic relationship between C5 (single MLG associated with isolates from Java plum in Brazil) and the MLGs of C1 (Pandemic biotype) presented in the minimum-spanning network. Java plum is native to India and Southeast Asia but has been widely introduced for its edible fruit and medicinal properties (Chaudhary & Mukhopadhyay, 2012). It was widely cultivated in Hawaii by the late 19th century, where it is now naturalized on all main islands and is considered invasive (Motooka, Castro, Nelson, Nagai, & Ching, 2003). By the early 20th century, it was cultivated in Bermuda, Cuba, Haiti, Jamaica, the French Islands of the Lesser Antilles and Trinidad and had been introduced to Florida, Puerto Rico and California (Kairo, Ali, Cheesman, Haysom, & Murphy, 2003; Morton, 1987). In Brazil, Java plum was introduced from India during Portuguese colonization in the 16th century and has become naturalized, and it is also found in Guatemala, Belize, Surinam and Venezuela. C5 (isolates from Java plum in Brazil) is closely related to C1 (Pandemic biotype) (F_{ST} = 0.26), and it is possible that the evolution of *A*. *psidii* on Java plum, which was widely planted in tropical/subtropical areas may have contributed to the emergence of C1 (Pandemic biotype). Of further interest is that a single, unique genotype of *A*. *psidii* was found on multiple myrtaceous hosts in South Africa, which was found to be distinct from the primary Pandemic genotype that was found in diverse global regions (Roux et al., 2016). The precise genetic relationships among the *A*. *psidii* genotype from South Africa and other global genotypes remain undetermined. Continued genotyping of additional material

collected from native and naturalized host species throughout the world is needed to better understand the potential pathways of spread, evolutionary relationships, and the invasive threat of this pathogen in general, and the Pandemic biotype.

A primary unanswered question is the potential source of the Pandemic biotype (C1/C4), which is distinct from the biotype associated with eucalypt/rose apple in Brazil/Uruguay (C2/C3) and the biotype associated with guava/Brazilian guava in Brazil (C6) (Graça et al., 2013). Limitations in sampling and genetic markers preclude further insights into the potential origin of the Pandemic biotype. Historical records and the relationship of MLGs from diverse geographic areas suggest that our collections of *A*. *psidii* isolates from this group likely all originated from areas where this biotype had invaded. Although natural occurrences and inoculation tests demonstrate that the Pandemic biotype has a broad host range (e.g., Carnegie & Lidbetter, 2012; Morin et al., 2012), little is known about native resistance to this biotype, which can allow inferences about the co-evolutionary history of this pathogen biotype with diverse hosts (Tobias, Guest, Külheim, Hsieh, & Park, 2016). Determining the source of an invasive forest pathogen is challenging because disease symptoms are frequently negligible in areas where hosts and pathogens have co-evolved. However, the continued collection and more robust genotyping of *A*. *psidii* from diverse hosts and global regions will provide further insights into genetic relationships and evolutionary history of *A*. *psidii* biotypes, which will allow further inferences about the source of the Pandemic biotype and other biotypes.

Fungi present unique challenges for microsatellite marker development due to poor library quality, low density of microsatellite loci across the genome, few loci suitable for primer design and low levels of polymorphism (Dutech et al., 2007), and *A*. *psidii* is no exception. In their review of data on microsatellite isolation from 37 fungal species from the literature, Dutech et al. (2007) found a mean of 8.6 loci used for analysis (comparable to the six loci used herein). Our study was limited by the modest number of markers that could be reliably amplified across all isolates coupled with the biotrophic nature of the pathogen and regulatory restrictions for invasive pathogens, which hampered the acquisition of adequate template for repeated genotyping attempts. Nevertheless, the six loci used in this study allowed a robust analysis of genetic diversity.

Previous attempts to assess the invasion potential of *A. psidii* (e.g., Booth & Jovanovic, 2012; Booth et al., 2000; Elith et al., 2013; Glen et al., 2007; Kriticos et al., 2013; Magarey et al., 2007; Roux et al., 2015) did not consider the existence of multiple biotypes. The discovery of multiple biotypes, each with unique host associations and climatic niches, raises quarantine and regulatory concerns. For example, USA-Hawaii, Australia, New Caledonia and Indonesia appear to have only the Pandemic biotype, and according to the MaxEnt bioclimatic model herein (Figures 5 and 6), may be at risk from the introduction of other biotypes. Conversely, the Pandemic biotype has not been found in Brazil or Uruguay, which appears to have suitable climate space for this biotype (Figure 4). Inoculation tests can help to assess threats posed by different pathogen biotypes, but environment likely plays a critical role in host susceptibility. Silva et al. (2014) showed that isolates of the C2/C3 biotype (eucalypt/rose apple–Brazil/Uruguay) were highly aggressive on Hawaiian ʻōhiʻa lehua plants, and Zauza et al. (2010) showed that diverse myrtaceous species from Australia varied greatly in their resistance/susceptibility to one race (UFV-02) of C2/C3 (eucalypt/rose apple–

Brazil/Uruguay) (Graça et al., 2011, 2013). When bioclimatic modelling is coupled with inoculation tests, it is clear that different biotypes pose distinct risks to various global regions, should they be introduced.

This study revealed several unique MLGs among widespread isolates that were associated with nine distinct genetic clusters, including two wide-ranging clusters (C1 and C4) associated with a Pandemic biotype. These results further demonstrate that genotypic diversity must be considered when assessing the invasive threat posed by this pathogen to myrtaceous hosts worldwide (Graça et al., 2013). Whether these genetic clusters represent cryptic species is yet to be determined. The use of more extensive molecular markers (Sandhu et al., 2016) may help to resolve the taxonomy, while also providing a stronger assessment for pathways of spread and areas at risk of invasion. Future studies that include isolates of *A. psidii* from additional geographic areas and hosts will also allow a better prediction of global geographic areas that are climatically suitable for *A*. *psidii* biotypes. Further, improving our knowledge of the roles of environmental factors and life history traits underlying the successful global spread of *A. psidii* is a key to prevent new invasions.

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