A unique genotype of the rust pathogen, *Puccinia psidii*, on Myrtaceae in South Africa

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Abstract The rust pathogen, *Puccinia psidii*, was first detected in South Africa in 2013 on a single non-native ornamental *Myrtus communis* tree. This prompted surveys of the country to determine its geographic distribution and host range. Previously developed microsatellite markers where used to characterize *P. psidii* isolates collected from these surveys. In addition, artificial inoculation studies and field observations were used to evaluate the susceptibility of native Myrtaceae to infection by *P. psidii*. The pathogen was found on native Myrtaceae in isolated natural situations and it was also common on exotic Myrtaceae in nurseries and gardens. Marker analysis showed that a single genotype of the rust is present in South Africa and that this

is different to the so-called “pandemic” strain recorded in countries outside Brazil. It was found to have a broad distribution with collections as far as 1500 km apart. The data provide firm evidence for a single introduction of the pathogen from an as yet unkown source. Its wide distribution, particularly in relatively isolated natural areas, suggests that *P. psidii* has been present in South Africa for much longer than implied by its first detection in the country.

Keywords *Eugenia natalitia*; Guava rust; Myrtle rust; *Myrtus communis*

Introduction

The rust pathogen, *Puccinia psidii* Winter (Urediniales, Sphaerophragmiaceae), has been described as “the greatest threat to the ecosystem” by Australian scientists and newspapers (Dayton and Higgins 2011; Glen et al. 2007; Pegg et al. 2014b). Subsequent to its first detection in Australia (Carnegie et al. 2010), *P. psidii* has led to the near extinction of 12 species of native Australian Myrtaceae (Carnegie et al. 2016; Pegg et al. 2014b). The pathogen has also had a significant impact on agriculture and commercial plantation forestry in other regions where it occurs, devastating the allspice (*Pimenta dioica*) industry in Jamaica (MacLachlan 1938) and resulting in considerable management costs to the eucalypt industry in Brazil (Alfenas et al. 2004; Ferreira 1983; Graça et al. 2011).

Puccinia psidii was first reported in 1884 from southern Brazil, infecting *Psidium guajava* (Winter 1884). The fungus has subsequently been reported

from North America (Marlatt and Kimbrough 1979) and Hawaii (Uchida et al. 2006), China (Zhuang and Wei 2011), Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2010), Africa (Roux et al. 2013), New Caledonia (Giblin 2013; Machado et al. 2015) and Indonesia (McTaggart et al. 2015). All of these reports, outside Brazil, were attributed to a single genoptype of *P. psidii* (Machado et al. 2015), dubbed the pandemic genotype (Ross-Davis et al. 2013). It is widely believed that *P. psidii* is native to Latin America due to its long history in that region and the fact that native Myrtaceae in the region are generally tolerant to infection, as opposed to the susceptible introduced species of Myrtaceae (Coutinho et al. 1998, Glen et al. 2007). Recent population genetic studies on collections of *P. psidii* from the Americas also suggest that host specific genotypes are native to Central and South America (Graça et al. 2013; Zhong et al. 2008, 2011).

For many years it was believed that *P. guajava* (guava) was the host of origin of *P. psidii* and that it underwent a host jump to non-native *Eucalyptus* and other Myrtaceae (Alfenas et al. 2005; Castro et al. 1983; Tommerup et al. 2003). However, a study by Graça et al. (2013) showed that isolates from guava and eucalypts in Brazil represented distinct genotypes/biotypes of the pathogen. The source population of the rust that occurs on eucalypts remains unknown although it seems likely that this would be in South or Central America.

Puccinia psidii has been reported from more than 73 genera and 450 species of plants in the Myrtaceae (Pegg et al. 2014b; Giblin and Carnegie 2014; Zauza et al. 2015). This plant family includes approximately 140 genera

accommodating about 5500 species and it occurs worldwide (Biffin et al. 2010). Three genera of the Myrtaceae occur in South Africa, namely *Eugenia*, *Metrosideros* and *Syzygium* (Coates Palgrave 2002). A closely related genus, *Heteropyxis*, is endemic to southern Africa and has been classified either in its own family, the Heteropyxidaceae, or a sub-family within the Myrtaceae (Stevens 2012; Wilson et al. 2005). Of the South African species of Myrtaceae, only *H. natalensis* has been tested for its susceptibility to *P. psidii*. Artificial inoculation trials in Brazil found that *H. natalensis* was highly susceptible to the genotype of rust fungus used in that study (Alfenas et al. 2005). Despite the relatively low number of native Myrtaceae in South Africa and other African countries, compared to Australia and South America, these plants are important components of the natural ecosystem, providing fruits for humans and animals, timber and medicinal compounds (Coates Palgrave 2002).

Puccinia psidii was first detected in South Africa in May 2013, on a single *Myrtus communis* tree, in a private garden on the south coast of KwaZulu-Natal (Roux et al. 2013). Subsequent to its detection, many articles have been published in garden and botanical magazines as well as in the media to raise awareness about the disease in South Africa (Roux 2015; Roux and Pegg 2014). Disease alerts and regular updates of Myrtle rust have also been provided to the South African forestry industry through newsletters and the web (http://fabinet.up.ac.za/archive). The aims of this study were to determine the distribution of *P. psidii* subsequent to its first report in South Africa; determine whether the pandemic genotype of *P. psidii* was present in South Africa by comparison with specimens from Australia using microsatellite markers, and

evaluate the relative susceptibility of native South African Myrtaceae to *P. psidii* by inoculation studies under greenhouse conditions.

Materials and methods

Disease surveys and sample collections

Surveys of *Eucalyptus* plantations, nurseries and native stands of Myrtaceae were conducted in 2013, 2014 and 2015 in order to assess the current distribution of *P. psidii* in South Africa. Sites were selected based on occurrence maps of native Myrtaceae (www.sanbi.org) published by the South African National Biodiversity Institute (SANBI) and as identified by Roux et al. (2015) as high risk areas for the occurrence of *P. psidii* in the country. These included areas along the coast of the KwaZulu-Natal Province, the Mbombela region in the Mpumalanga Province and the Tzaneen and Soutpansberg regions in the Limpopo Province. Reports of possible infected plants in private gardens, made by the general public, were investigated after awareness campaigns in the media and garden forums. Eucalypt nurseries and plantations in high risk areas were visited on an *ad hoc* basis, and monitored by forestry staff.

Fragment analyses and genotyping

Seven *P. psidii* samples collected from countrywide surveys, as well as four isolates from Australia, were used in the genetic analyses (Table 1). Urediniospores were harvested from infected leaf samples using a vacuum pump and stored in 2mL screw- cap vials at -80°C prior to DNA extraction. The genotypes of samples were determined using seven microsatellite markers (PpSS012, PpSS014, PpSS018, PpSS022, PpSS102, PpSS161 and PpSS195) developed by Zhong et al. (2008) and modified by Graça et al. (2013). Genomic DNA was extracted from a single uredinium per host using the Ultraclean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, USA). PCR mixtures included 1x PCR FastStart Taq Buffer with $MgCl_2$ (Sigma-Aldrich, St. Louis, Missouri, USA), 200 μM dNTPs, 0.1 μM forward (labeled with NED™, FAM™, PET® or VIC™ fluorescent dye) and reverse primers, 1 unit FastStart Taq DNA polymerase (Sigma-Aldrich), and DNA template in 12,5 μl reaction volumes. PCR amplifications were made using a Veriti® Thermal Cycler (Life Technologies, Carlsbad, California, USA) with one cycle at 95°C for 5 min, followed by three cycles at 95°C for 30s, 52–56°C (depending on the primer pair/microsatellite marker) for 30s, 72°C for 80s, 35 cycles at 94°C for 15s, 52–56°C (depending on the micrprimer pair/microsatellite marker) for 15s and 45s at 72°C. Fragment analyses were performed using an ABI Applied Biosystems 3500xl sequencer (Thermo Fisher Scientific, Carlsbad, USA) at the Sequencing Facility of the Faculty of Natural and Agricultural Science, University of Pretoria. Alleles were scored and sizes

determined using GeneMapper® Software Version 4.1 (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA).

Table 1 *Puccinia psidii* samples used for microsatellite analyses.

Host – South Africa	**Origin**
Backhousia citriodora	Private Garden, Irene, Gauteng
Eugenia erythrophylla	Nursery, Port Edward, KwaZulu-Natal
Eugenia natalitia	Natural forest, Grootbos, Modjadjiskloof, Limpopo
E. natalitia	Natural forest, Wolkberg, Limpopo
Heteropyxis natalensis	Nursery, Port Edward, KwaZulu-Natal
Myrtus communis	Private Garden, Pennington, KwaZulu-Natal
M. communis	Nursery, Tshwane, Gauteng
Host – Australia	
Backhousia citriodora	The Channon, New South Wales
Gossia inophloia	Salisbury, Queensland
Melaleuca viminalis	Chapel Hill, Queensland
Rhodamnia rubescens	Chapel Hull, Queensland

Inoculation studies

Native South African Myrtaceae were sourced from nurseries in the Gauteng, KwaZulu-Natal and Limpopo Provinces and acclimatized to greenhouse conditions at the University of Pretoria prior to inoculation with *P. psidii*. Five plants each of six native species (*Eugenia erythrophyllum*, *E. verdoornii*, *Heteropyxis canescens*, *H. natalanesis*, *Syzygium cordatum*, *S. legatii*) were

obtained for inoculation studies. Four weeks prior to inoculation, branch tips were pruned back to produce even aged, new-growth shoots that would be most susceptible to infection.

Inoculum was sourced from a natural *P. psidii* infection on *Eugenia natalitia* seedlings near Tzaneen in the Limpopo Province. Spores were harvested with a fine-haired paint brush and suspended in 1mL of sterile distilled water (SDW) with 0.05% Tween 20. This mixture was applied to new leaves of *Syzygium jambos* for propagation of the spores. The plants were watered and covered with clear plastic bags in a glasshouse for 48hrs to maintain humidity and facilitate infection, then grown under glasshouse conditions at ~25 ^{o}C for two weeks. Inoculations were done so that the first 12hrs after inoculation consisted of night time and thus dark conditions for spore germination. The spores were harvested using a vacuum pump, desiccated for 5–7 days, and stored at -80^{o}C.

Desiccated *P. psidii* urediniospores were removed from -80^{o}C storage and added to sterile distilled water (SDW) with 0.05% Tween 20. The spore suspension was incubated at room temperature to facilitate rehydration of the spores and mixed to ensure an even distribution of the inoculant. Spore counts were made using a haemocytometer and the suspension adjusted to a concentration of 1 x 10^{5} spores/ml. Native plants were inoculated with a fine mist of the spore suspension using an artist airbrush (Iwata LPH-80) powered by a 1/6 HP tubular compressor (Iwata IS 875HT Smart Jet Plus). The inoculum was applied to both the abaxial and adaxial leaf surfaces until just before the point of run off using the method described by Pegg et al. (2014a).

Syzygium jambos plants were included as indicators of positive infection. The plants were assessed for symptoms after 14 days and rated for disease resistance based on the scale developed by Junghans et al. (2003a).

Results

Disease surveys and sample collections

Puccinia psidii was present in the Gauteng, KwaZulu-Natal and Limpopo Provinces of South Africa (Fig. 1). In all areas, both uredinia and telia were common (Fig. 2). *Puccinia psidii* was common on non-native *Myrtus communis* in commercial ornamental nurseries that stocked this plant in the Gauteng Province (four nurseries). The only other report from a non-native host was from a *Backhousia citriodora* plant found in a private garden in Gauteng.

New reports from KwaZulu-Natal (KZN) include one from a private garden in the town of Melmoth in eastern KZN, from *M. communis*, and a second from native Myrtaceae in a private nursery on the south coast of the province. Infected hosts in the nursery included *Eugenia erythrophylla, E. natalitia, E. umtamvunensis* and *Heteropyxis natalensis* (Table 2). During two previous visits to the nursery in 2013 and 2014, no rust had been observed on these plants, but during March of 2015 the disease was found on several plants of each of the species.

In the Limpopo, *P. psidii* was found only on native *E. natalitia* and only in a natural forest. The first discovery of the pathogen in the Province was in the

Wolkberg Wilderness area near Tzaneen, on two plants. Subsequent visits to the region revealed infections of the fungus at a number of other sites, all in native forest patches and only on *E. natalitia*. The pathogen was also found on *E. natalitia* at two sites in the Soutpansberg Mountains, i.e. at the western most point of the mountain range near the town of Vivo and at the eastern most part of the range near the city of Thohoyandou (Fig. 1).

No reports of possible *Puccinia psidii* symptoms on plantation *Eucalyptus* species in South Africa were received during the period 2013-2015. Visits to commercial plantation forestry nurseries in the same period also did not result in the discovery of *P. psidii* on eucalypt seedlings, cuttings or hedges.

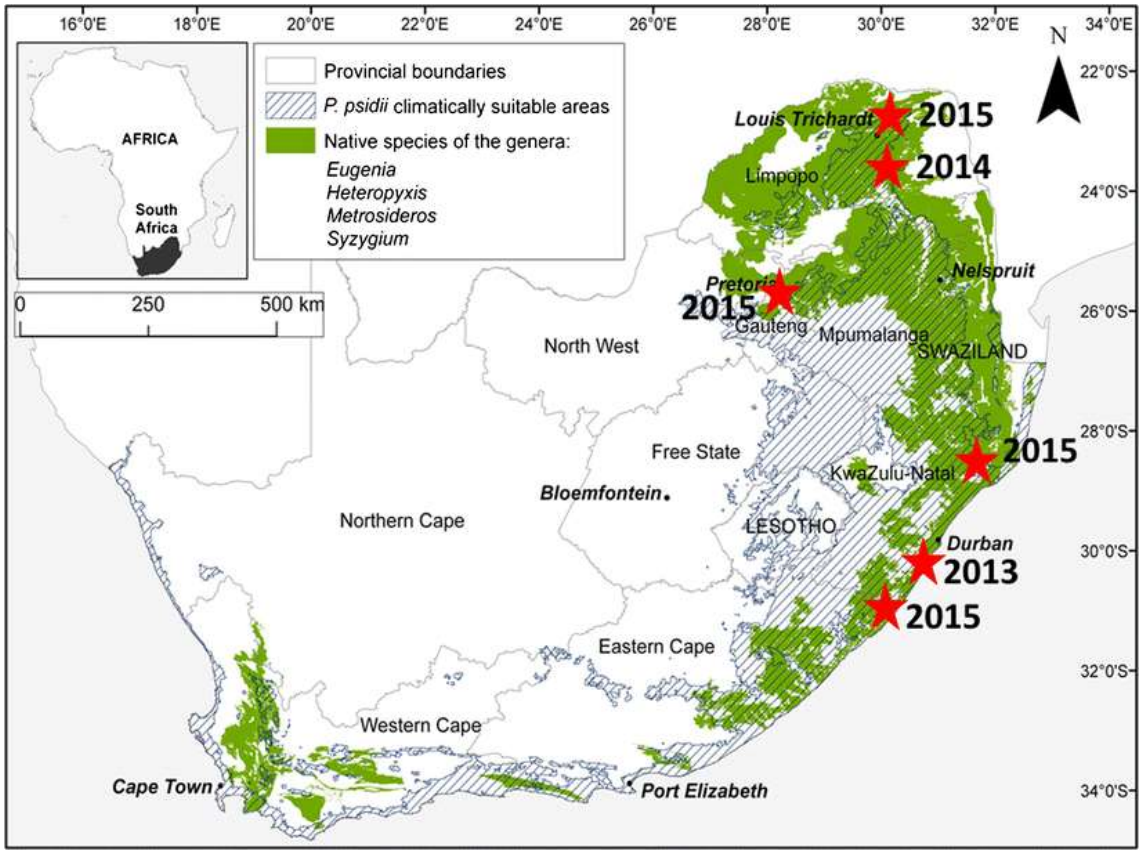

Fig. 1. Map of South Africa showing areas from which *Puccinia psidii* has been confirmed (Base map produced by Dr. Ilaria Germizhuizen, ICFR, South Africa).

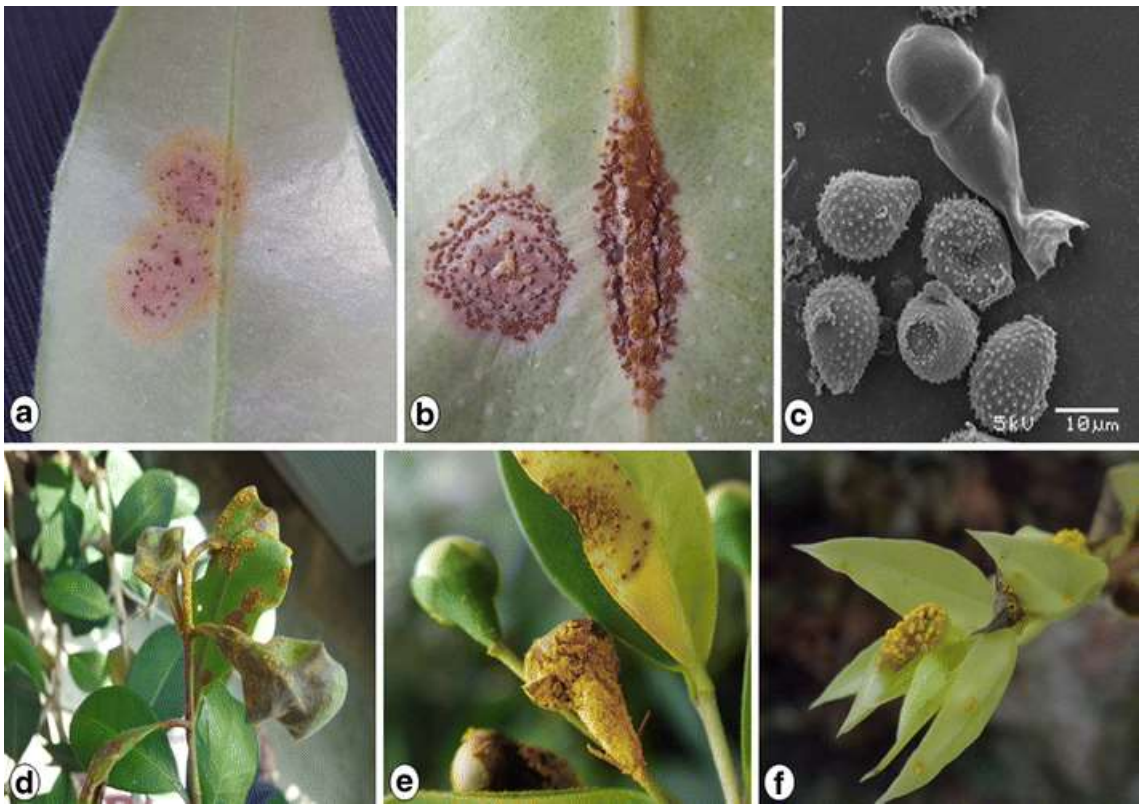

Fig. 2. Typical symptoms of *Puccinia psidii* infection on Myrtaceae in South Africa. (A) Leaf spots with yellow masses of uredinia and rust brown telia on *Eugenia erythrophyllum*, (B) telia on *E. erythrophyllum*, (C) teliospore and urediniospores on a *Myrtus communis* leaf, (D) dying leaves and shoot of *E. erythrophyllum* covered with masses of yellow uredinia, (E) infected flower buds of *Backhousia citriodora*, (F) young infect shoots of *E. umtamvunensis* with uredinia.

Table 2 Native South African Myrtaceae and Heteropyxidaceae[a].

Taxon	Conservation status[b]	Susceptible to *P. psidii*
Eugenia albanensis	Least concern	Unknown
E. capensis subsp. a	Least concern	Unknown
E. capensis subsp. capensis	Least concern	Unknown
E. capensis subsp. gueinzii	Least concern	Unknown
E. erythrophylla	Near threatened	Yes
E. natalitia	Least concern	Yes
E. pusilla	Extinct	Unknown
E. simii	Vulnerable	Unknown
E. umtamvunensis	Endangered	Yes
E. uniflora	Unknown	Unknown
E. verdoorniae	Near threatened	Yes
E. woodii	Least concern	Unknown
E. zeyheri	Least concern	Unknown
E. zuluensis	Least concern	Unknown
Heteropyxis canescens	Least concern	Yes
H. dehniae	Least concern	Unknown
H. natalensis	Least concern	Yes
Metrosideros angustifolia	Least concern	Unknown
Syzygium cordatum	Least concern	Yes
S. gerrardii	Least concern	Unknown
S. guineense subsp. barotsense	Least concern	Unknown
S. guineense subsp. guineense	Least concern	Unknown
S. intermedium	Least concern	Unknown
S. legatii	Least concern	Yes
S. pondoense	Rare	Unknown

[a]New host records, not in Giblin and Carnegie Global host list.

[b]Conservation status as given by the South African National Biodiversity Institute (SANBI) – based on national status as classified by the National Environmental Management Biodiversity Act (NEMBA) regulations on Threatened and Protected Species in South Africa. This is not based on threat posed by *P. psidii.*

Fragment analyses and genotyping

Isolates used for fragment analyses were collected across three provinces (Gauteng, Limpopo and KwaZulu-Natal) and included isolates from *Backhousia citriodora* and *Myrtus communis* (Gauteng), *Eugenia natalitia* (Limpopo), *Eugenia erethrophylla*, *H. natalensis* and *M. communis* (KZN). Four samples of *P. psidii* from Queensland, Australia were included for comparison with the South African material (Table 1). The seven *P. psidii* samples from South Africa had identical alleles at all seven microsatellite loci. Six of the loci contained two alleles each while locus PpSS195 was homozygous. The single multilocus genotype (MLG) for South Africa was different to that of the single MLG obtained from the four Australian isolates of *P. psidii* (Table 3).

Table 3 Allele sizes of microsatellite markers of *Puccinia psidii* from South Africa and Australia using primers modified by Graça *et al.* (2013).

Alleles	Australia	South Africa
PpSS012	230-236	234-237
PpSS014	207-211	205-213
PpSS018	170-172	165-174
PpSS022	158-160	149-154
PpSS102	140	140-143
PpSS161	276-290	269-288
PpSS195	214	212

Inoculation studies

Of the six native South African species of Myrtaceae tested, *Eugenia erythrophylla* was the most susceptible, and all seedlings of this species showed very high levels of infection. *Eugenia verdoorniae* was also highly susceptible and all seedlings of this species developed uredinia. Some uredinia developed on *Syzygium legattii* and *H. canescens*, but these species appeared to have a relatively high level of tolerance to infection. *Syzygium cordatum* appeared resistant to the South African genotype of *P. psidii* under glasshouse conditions (Table 4).

Table 4 Average infection scores of *Puccinia psidii* on selected South African Myrtaceae 14 days post inoculation under greenhouse conditions*.

Myrtaceae species	**Average infection score**
Eugenia erythrophylla	5
E. verdoorniae	4
Heteropyxis canescens	2.6
H. natalensis	3
Syzygium cordatum	1
S. legattii	2.5
S. jambos (positive control)	5

*Rated based on Junghans et al. 2003a

Discussion

Puccinia psidii was first discovered in South Africa on a single, non-native ornamental plant in a garden (Roux et al. 2013). Early efforts to find additional sources of infection in that region failed. However, as surveys have expanded and with the help of public awareness campaigns, the rust has been found on numerous host plants and it is clearly widely distributed in South Africa. The results of this study have thus shown for the first time that *P. psidii* occurs under natural conditions on a number of native species of Myrtaceae. While it has also been found on various non-native Myrtaceae in gardens and nurseries, it is interesting that it has yet to be detected on species of *Eucalyptus* that are widely established in South African plantations.

An important result of this study was that all the isolates of *P. psidii* from South Africa represented a single genotype of the pathogen based on seven microsatellite markers. The isolates tested included those from both native and non-native plants and collections were as much as 1500km apart. These results show that there was possibly one introduction of *P. psidii* into South Africa, and it may have been present in natural environments much earlier than first detected.

The genotype of *P. psidii* in South Africa was found to be different to the one currently known in Australia. Previous studies have shown that the Australian genotype is identical to that found in Indonesia (McTaggart et al. 2015), New Caledonia, China (Machado et al. 2015) and Hawaii (Machado et al. 2015; Sandhu et al. 2015). Several genotyping studies using a variety of

microsatellite markers have traced the orgin and global movement of collections of *P. psidii* (Graça et al. 2011; Machado et al. 2015; Ross-Davis et al. 2013; Zhong et al. 2011). These previous studies have identified a "pandemic" genotype that is clonal, and that has a wide host and geographic range in the Caribbean, Mexico and the USA (Ross-Davis et al. 2013). The "pandemic" genotype is distinct from the MLGs found in Brazil (Graça et al. 2013; Ross-Davis et al. 2013). What is clear at this stage is that the South African genotype is different to the one occurring in Hawaii and Australia (pandemic genotype) and thus, neither of these genotypes has a known source. It is most likely that in both cases the source material for infection would be a plant or plants in South or Central America. Resolution of this important question will require field surveys and additional collections in that area.

In South America where *P. psidii* is most likely native, multiple host-associated MLGs have been found (Aparecido et al. 2003; Graça et al. 2013; Machado et al. 2015). In Hawaii and Australia, by contrast, a single genotype that infects multiple hosts occurs (Carnegie and Lidbetter 2012; Graça et al. 2013; Loope 2010; Sandhu et al. 2015; Zhong et al. 2011), indicative of an introduced pathogen. Additionally, the genetic diversity of *P. psidii* in Brazil is reported to be much higher than that in Australia and Hawaii (Graça et al. 2013; Machado et al. 2015). The same situation is found in South Africa, where multiple native and non-native genera and species of Myrtaceae are affected by a single *P. psidii* genotype.

Telia were abundant on infected plants in South Africa, both on native and non-native hosts. This is in contrast to reports from other countries where they

are reported to be rare (Glen et al. 2007; Pegg et al. 2014b; Perez et al. 2011). The presence of abundant telia in South Africa could result in recombination and generation of novel genotypes in the country. Telia may also facilitate the survival (overwintering) of the pathogen, allowing for rapid population build-up, after situations not suitable for development of uredinia. Recent studies have shown there are mutations, but no recombination in Australian populations of *P. psidii* (Machado et al. 2015). Future studies with additional isolates of *P. psidii* and microsatellite markers, will be required to investigate the diversity and if recombination has occurred in populations of the South African genotype.

Puccinia psidii has a very wide distribution in South Africa including native and non-native Myrtaceae hosts. This extensive distribution suggests that it has been present in the country for substantially longer than the three years since its first report in the country. It seems most likely that the pathogen has been distributed widely across South Africa with the nursery trade. *Myrtus communis*, the host on which it was first detected (Roux et al. 2013), is highly susceptible to infection. *Puccinia psidii* infection was found in every visited nursery that sold this plant in the greater Tshwane and Ekhuruleni Municipal areas of Gauteng Province. Some nursery owners reported that *M. communis* plants had died prior to 2013 and that this had resulted in the plant being abandoned from production programmes. The trade in ornamental plants and "plants for planting" has been implicated as a major pathway in the spread of plant pathogens globally (Liebhold et al. 2012; Palm and Rossman 2003). This has also resulted in calls for bans in the trade of detrimental ornamental plants and alternative global strategies in a bid to reduce the spread of insect pests

and pathogens (Anonymous 2011; Wingfield et al. 2015).

Field surveys and artificial inoculation studies conducted as part of this study have substantially increased the known host range of *P. psidii* in South Africa. Under natural conditions, in nurseries and in forests, the host range of *P. psidii* in the country now includes five species of native Myrtaceae and two non-native species. Under greenhouse conditions, and using artificial inoculations, an additional three native Myrtaceae and one non-native (*S. jambos*) were successfully infected with the South African genotype of *P. psidii*. Amongst the susceptible native Myrtaceae, two (*E. erythrophylla*, *E. verdoorniae*) are near threatened species and one (*E. umtamvunensis*) is endangered. All three of these *Eugenia* species are endemic to South Africa and occur in a very small area known as Pondoland in the southern part of the KwaZulu-Natal Province, stretching into the Transkei area of the Eastern-Cape Province. Of those tested under greenhouse conditions, *E. erythrophylla,* was highly susceptible. Equally susceptible was the commonly used indicator plant *S. jambos*. Although infections occurred on *S. cordatum*, levels were very low for this wide spread African Myrtaceae species.

The Myrtaceae comprise 17 tribes and an estimated 5500 species (Biffin et al. 2010). The *P. psidii* genotype discovered in South Africa infects hosts in the Myrteae, Syzygieae, Backhousieae and Psiloxyloideae. This suggests that it may have the potential to infect many other hosts in the sub-family Myrtoideae, including commercially important eucalypts and *P. guajava*. Morin et al. (2012), conducted extensive host range studies of Australian Myrtaceae and concluded that there is no apparent association between the presence or absence of *P.*

psidii disease symptoms and the phylogenetic relatedness of host taxa. Furthermore, they showed that all Australian Myrtaceae species have the potential to become infected with *P. psidii* to some degree. The occurrence of resistance (*R*) genes in eucalypts (Junghans et al., 2003b; Mamani et al., 2010), however, suggests the retention of ancient *R*-genes (Tobias et al. 2015) and bodes well for breeding programmes seeking to produce disease tolerant plants for commercial purposes. It would serve the commercial plantation industry and guava growers well to screen their breeding populations for resistance to *P. psidii* and thus to avoid future costly losses due to rust. However, preservation of native species in natural ecosystems that might be threatened by *P. psidii* would be much more complex and serious losses to native biodiversity could ermerge as is now being experienced in Australia.

Acknowledgements We are grateful to Dave and Audrey Woods of Indigiflora, Munster, for providing us with plants to use in the screening trials and to Mr. Jason Batemen of the Manie van der Schijff Botanical Gardern (University of Pretoria) for assistance with sourcing plants. Prof. Braam van Wyk (Department of Plant Sciences, University of Pretoria) provided valuable discussions regarding the taxonomy of Myrtaceae and Dr. Geoff Pegg (Department of Agriculture, Fisheries and Forestry, Horticulture and Forestry Science, Agri-Science Queensland, Australia) is thanked for provision of specimens from Australia. This work is based on research partially supported by the Tree Protection Co-operative Program (TPCP) and the National Research Foundation of South Africa (Grant specific unique

reference numbers UID 78566 and UID 83924). The grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are that of the authors and that the NRF accepts no liability whatsoever in this regard.

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