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Evidence that *Austropuccinia psidii* may complete its sexual life cycle on Myrtaceae

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The rust fungus *Austropuccinia psidii* has spread globally and naturalized in areas with naïve species of Myrtaceae. Previous studies have revealed multiple strains of *A. psidii* within South America and two strains outside of its native range. The rust spreads by windborne mitotic urediniospores, which are the dominant spore stage. Teliospores and basidiospores of *A. psidii* are also formed; however, the biological role of these stages in the life cycle is unknown. Experiments presented here tested whether basidiospores of *A. psidii* could infect *Syzygium jambos*. The sori produced by infection with basidiospores were screened with five microsatellite markers to confirm whether they were a product of recombination. The findings showed that basidiospores of *A. psidii* could cause infection on species of Myrtaceae and the resulting sori were a product of recombination. This has important implications for programmes that breed for resistance to this aggressive pathogen in commercial eucalypt forestry.

Keywords: eucalyptus rust, guava rust, myrtle rust, *Puccinia psidii*, Pucciniales, Uredinales

Introduction

Austropuccinia psidii (syn. *Puccinia psidii*, Sphaerophragmiaceae, Pucciniales) causes rust on approximately 460 species in 73 genera of Myrtaceae (Giblin & Carnegie, 2014; Roux *et al.*, 2016; Beenken, 2017). These hosts include species of *Eucalyptus* that are important to the forestry industry (Coutinho *et al.*, 1998), and endangered species in genera such as *Eugenia*, *Gossia* and *Rhodamnia* (Glen *et al.*, 2007; Pegg *et al.*, 2014; Carnegie *et al.*, 2015; Roux *et al.*, 2016). *Austropuccinia psidii* probably originated in South America and spread globally through North and South America (Marlatt & Kimbrough, 1979; Rodas *et al.*, 2015), northern Asia (Kawanishi *et al.*, 2009; Zhuang & Wei, 2011), the Pacific region (Uchida *et al.*, 2006; Carnegie *et al.*, 2010), South Africa (Roux *et al.*, 2013, 2016) and Southeast Asia (McTaggart *et al.*, 2015; du Plessis *et al.*, 2017).

Austropuccinia psidii has three known life cycle stages: (i) a mitotic, dikaryotic uredinial stage, which can be used to distinguish it from other rusts on Myrtaceae (Maier *et al.*, 2015), (ii) a telial stage with diploid teliospores (Morin *et al.*, 2014), and (iii) basidiospores that develop on a basidium and have either one or two nuclei (Morin *et al.*, 2014). Basidiospores are necessary for sexual reproduction in *A. psidii* because this stage contains recombinant DNA produced by meiosis in the teliospores. The environmental conditions for production of each spore stage were studied in Brazil. Uredinia formed at temperatures of 15–20 °C (Aparecido, 2001; Blum & Dianese, 2001), telia were produced at warmer temperatures of 21–25 °C, and basidiospores were optimally produced at 21 °C, with between 400–600 basidiospores per square mm of telia (Aparecido, 2001).

The life cycle of *A. psidii* was summarized by Glen *et al.* (2007), with the role of basidiospores based on the work of Figueiredo (2001; cited in Glen *et al.*, 2007). The role of teliospores and basidiospores in the life cycle of *A. psidii* is controversial. Morin *et al.* (2014) observed that over 3000 germinated basidiospores did not penetrate the cell walls of host plants. Pustules that formed from inoculations showed no sign of recombination in their study and they attributed successful inoculations with basidiospores, also made by Figueiredo *et al.* (1984; cited in Morin *et al.*, 2014), to incidental infections from 'rogue' urediniospores that sometimes occur in telia. The current understanding of the life cycle of *A. psidii* is that basidiospores of the strain present in Australia most probably do not cause infection on Myrtaceae (Morin *et al.*, 2014). Consequently, *A. psidii* is considered a clonal pathogen spread by its mitotic urediniospores. Morin *et al.* (2014) and Glen *et al.* (2007) both hypothesized *A. psidii* as autoecious, being able to complete its life cycle on one host. A heteroecious hypothesis was suggested by Simpson *et al.* (2006), who proposed *A. psidii* may have unknown spermogonial and aecial stages that occur on an unrelated host plant.

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There are several host-associated strains of *A. psidii* on native plants in Brazil, Colombia, Jamaica and Uruguay (Graça *et al.*, 2013; Ross-Davis *et al.*, 2014; Granados *et al.*, 2017). A pandemic strain, with an unknown origin, occurs on a wide host range in Australia, California, Central America, China, Colombia, Hawaii, Indonesia and New Caledonia (Graça, 2011; Ross-Davis *et al.*, 2014; Machado *et al.*, 2015; McTaggart *et al.*, 2015; Granados *et al.*, 2017). A different strain occurs on several hosts in South Africa (Roux *et al.*, 2016). The known strains of *A. psidii* are currently considered clonal, and allelic diversity within a population has been attributed to mutations. For example, a study on populations of *A. psidii* in Australia found new genotypes within 5 years after its arrival, and these were attributed to mutations in microsatellite loci rather than recombination events (Machado *et al.*, 2015).

The telial stage of *A. psidii* is commonly observed in South Africa (Roux *et al.*, 2016). From an evolutionary perspective, it would not be advantageous to the survival of the species if this spore stage was nonfunctional. This would be particularly true if telia were produced in response to stressful conditions, such as extreme temperatures or dry periods. The hypothesis tested in the present study is that sexual recombination may play a role in the life cycle of *A. psidii*. This was assessed by whether basidiospores caused infection on a species of Myrtaceae, and whether there was evidence for recombination, which was tested using microsatellite markers.

Materials and methods

Parental genotype

A single genotype of *A. psidii* was found in South Africa in a previous study (Roux *et al.*, 2016). Urediniospores from a single uredinium of this genotype were inoculated on *Syzygium jambos*, a susceptible host, and bulked for production of telia following the method of Roux *et al.* (2016). Three single uredinia from this bulked inoculation were confirmed as being the same genotype obtained by Roux *et al.* (2016) using microsatellite markers.

Production of basidiospores

Leaves of *S. jambos* that were inoculated with urediniospores of the South African genotype of *A. psidii* and that bore telia, were collected and placed in a humid container at 16–23 °C for 48 h. The telia were examined under a dissection microscope to observe germination of basidia. Telia that germinated were selectively scraped from the leaf surface with a scalpel to avoid uredinia, and placed in sterile distilled water with 0.05% Tween 20. This suspension was then gently vortexed to separate clumps of telia.

Separation of basidiospores from teliospores and urediniospores

Based on the spore sizes reported by Shivas *et al.* (2014), basidiospores (8–11 μm diam.) were separated from teliospores (23–50 × 14–28 μm) and urediniospores (17–21 × 13–17 μm) by filtration through a filter membrane with 8 μm pores (Macherey-Nagel). The spore suspension was vacuumed through the filter membrane, and the filtrate centrifuged for 2 min at 3000 ***g*** to concentrate spores. The filtrate was examined under a light microscope to determine the concentration of basidiospores (per μL) and to make sure that only basidiospores were present.

Inoculation of host tissue with basidiospores

Two repeats of the experiment were performed with suspensions of basidiospores at either eight or 25 basidiospores μL^{-1}. The suspensions of basidiospores were pipetted in droplets of 3 μL on six to eight developing or unopened leaves of *S. jambos*. One plant was inoculated at a time due to the low concentration of basidiospores. A second plant was mock inoculated with sterile water with 0.05% Tween 20 as a control. Plants were sprayed with distilled water, covered with plastic bags for 48 h to maintain leaf wetness, and kept in a phytotron at 18–23 °C.

DNA extraction and microsatellite analysis of single sori produced from infection

Individual sori that developed after inoculations with basidiospores were excised with a sterile scalpel and DNA was extracted with a MoBio Microbial DNA extraction kit (QIAGEN). DNA was extracted from 11 single telia in the first experiment and four telia or uredinia in the second experiment. Five microsatellite markers that amplified heterozygous loci for the strain of *A. psidii* present in South Africa (Roux *et al.*, 2016) were used to determine the multilocus genotypes of sori produced from the inoculations with basidiospores. These microsatellite markers were originally developed by Zhong *et al.* (2008), modified by Graça *et al.* (2013) and amplified following the methods used by Roux *et al.* (2016). Microsatellites were analysed using an ABI Applied Biosystems PRISM 3500xl (Life Technologies) at the Sequencing Facility of the Faculty of Natural and Agricultural Science, University of Pretoria. Allele sizes were determined using the Liz500 (-250) size standard and scored with GENEMAPPER v. 4.1 (Applied Biosystems). Alleles produced by markers PP_14, PP_18 and PP_102 were scored at a relative fluorescence unit (rfu) range between 200–1000 due to the low concentration of PCR product, while those for PP_22 and PP_161 were scored at an rfu of 1000 or greater. The allelic profiles of all scored alleles and individuals are provided in Figure S1.

Test for recombination

Evidence for recombination was tested by whether any of the individuals obtained from sori that developed after inoculation with basidiospores differed in their multilocus profile to that of the parents at five microsatellite loci. For example, homozygous genotypes were expected at some of the loci that were heterozygous in the parents.

Number of nuclei and germination of basidiospores

Morin *et al.* (2014) found that basidiospores of *A. psidii* contained two nuclei. This was retested in the current study using the DAPI stain protocol for fresh spores described by Morin *et al.* (2014). Stained nuclei were examined at ×100 magnification using a Zeiss Axioskop fluorescence compound microscope. A 5 μL aliquot of the basidiospore suspension was incubated on

a microscope slide placed on a Petri dish with 1.5% water agar under the same temperature conditions as those for the inoculation experiments. Germination of basidiospores was assessed after 24 h.

Results

Separation of basidiospores from other spore stages

It was possible to reliably separate basidiospores from teliospores and urediniospores with the applied filtration method. Teliospores and urediniospores were not observed in the filtrate prepared from a suspension of harvested basidiospores, telia and uredinia. The first experiment yielded a concentration of 25 basidiospores μL^{-1} and the second experiment eight basidiospores μL^{-1}.

Infection of host tissue by basidiospores

Symptoms, such as chlorosis and leaf bending, developed 14 days post-inoculation. Sori of either telia or uredinia developed after 18 days. Symptoms only developed on one leaf of each plant in the two experiments, and the control inoculations were free of symptoms.

Microsatellite marker analysis of infections caused by basidiospores

A total of 15 sori, 11 samples from the first replicate and four samples from the second replicate, were screened with five microsatellite markers (Fig. 1). Of the 64 alleles scored, 28 were homozygous and 36 were heterozygous at five loci (Table 1; Fig. S1). Not all markers were amplified for all samples, possibly due to low DNA concentrations. These missing data were recorded in 11 instances.

Test for recombination

Recombination was directly observed as 11 multilocus genotypes were recovered that differed from the parental genotype. The ratio of homozygous loci was 0.44. The nonparental genotypes and observed homozygotes supported evidence for recombination in the sampled progeny.

Number of nuclei and germination of basidiospores

The spore stages stained with DAPI had the same number of nuclei reported by Morin *et al.* (2014). Basidiospores had two nuclei per cell (Fig. S2). Urediniospores were dikaryotic and teliospores had one large nucleus. Basidiospores germinated with two polar germ tubes.

Discussion

The results of this study support the hypothesis that basidiospores of *A. psidii* are capable of infection on Myrtaceae. This was demonstrated in a controlled experiment by infections caused from basidiospores. Subsequent tests on sori caused by these infections using microsatellite markers showed that recombination had occurred. There are no studies that have clearly shown that natural infections of *A. psidii* are the products of recombination.

Newly emerged leaves of *S. jambos* were infected with pure suspensions of basidiospores. Morin *et al.* (2014) concluded that rogue urediniospores contaminated inoculation attempts made with basidiospores. This potential problem was eliminated in the present study by removal of urediniospores from the inoculum with size-dependent filtration.

The second part of the present study confirmed that sori produced by successful inoculations with basidiospores were the progeny of recombination in teliospores. This is in contrast to infection by mitotic urediniospores. Evidence for recombination was observed by the homozygous loci in the progeny that were heterozygous in the parents, and by 13 progeny that had multilocus genotypes different to the parent. These results indicated the infections were not from clonal spores.

The conclusions made in this study hinge on loci scored as homozygous for the amplified microsatellites.

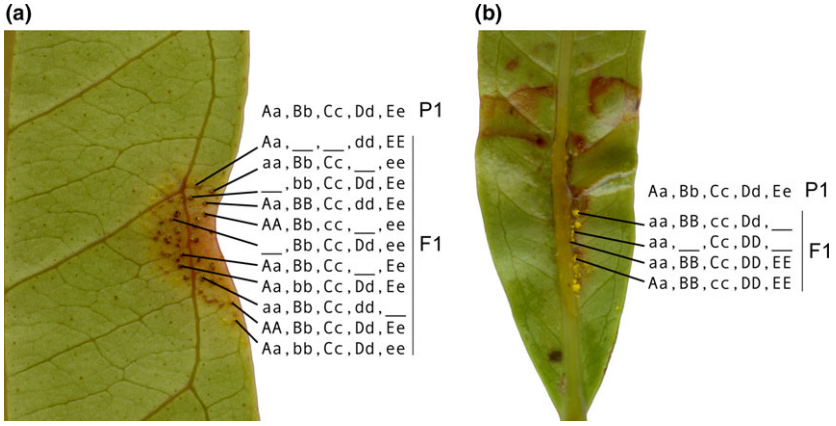

Figure 1 Alleles scored for individual sori of *Austropuccinia psidii* at five microsatellite loci from an inoculation with basidiospores. (a) First experiment; (b) second experiment. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Alleles from five microsatellite markers scored for sori of *Austropuccinia psidii* formed from infection by basidiospores

	Marker									
Experiment_Pustule	PP_18		PP_102		PP_22		PP_14		PP_161	
Parent (from 9 isolates)	165	174	140	142	150	154	205	213	270	288
01_01	165	174*	NA		NA		213	213	270	270
01_02	174	174*	140	142	150	154	NA		270	288
01_03	NA		142	142	150	154	205	213*	270	288
01_04	165	174	140	140*	150	154	205	213*	270	270*
01_05	165	165*	140	142*	154	154	NA		270	270*
01_06	NA		140	142*	150	154	205	213*	270	288
01_07	165	174*	140	142	150	154	NA		270	288
01_08	165	174	142	142	150	154	205	213	270	288
01_09	174	174	140	142	150	154	205	205	NA	
01_10	165	165	140	142	150	154	205	213	270	288
01_11	165	174	142	142	150	154	205	213	288	288
02_01	174	174	140	140	154	154	205	213	NA	
02_02	174	174	NA		150	154	205	205	NA	
02_03	174	174	140	140	150	154	205	205	270	270
02_04	165	174	140	140	154	154	205	205	270	270

Heterozygotes are shaded light grey, homozygotes shaded dark grey, and NA is used for instances of no amplification of PCR product. Allelic profiles that were below 1000 relative fluorescence units are marked with an *; the profiles are provided in Figure S1.

There were data missing for 11 of these 75 amplifications, which might be interpreted as evidence for PCR bias or null alleles at the microsatellite markers. However, the unsuccessful amplifications were attributed to low quantities of DNA rather than PCR bias or null alleles. This was because there was no bias when nine of ten alleles were amplified as homozygotes. In addition, there was a low chance of mutation in the binding sites of primers after one generation, and this would rule out the possibility of null alleles (Chapuis & Estoup, 2007). The fact that these are true homozygous loci provides the best explanation for the observed results.

Results from the present study have supported the hypothesis that *A. psidii* is autoecious, with basidiospores that infect the same hosts on which telia and uredinia are formed. This may be cause to reject the hypothesis that *A. psidii* is heteroecious with an unknown aecial host. Taxa in Sphaerophragmiaceae, which include species of *Dasyspora*, *Puccorchidium*, *Sphaerophragmium* and *Sphenorchidium*, as well as *A. psidii*, appear to have autoecious life cycles (McTaggart *et al.*, 2016b; Beenken, 2017).

The life cycle of *A. psidii* hypothesized in this study possibly begins with the anastomoses of hyphae produced from basidiospores. The anastomoses may form a dikaryotic hymenium, which produces telia or uredinia. This indicates that a uredinial stage need not be formed if environmental conditions suit the production of telia. Uredinia are mitotic, which was supported by three identical multilocus genotypes from infections made with urediniospores. Telia can form from uredinia, or form separately to uredinia. Teliospores are diploid and germinate to form a basidium. Basidiospores, which are produced on the basidium, contain one or two nuclei, and two polar germ tubes are formed from each basidiospore in the South African strain. The basidiospores examined by Morin *et al.* (2014) had one germ tube. This may indicate intraspecific variation between the pandemic and South African strains of *A. psidii*.

De Backer *et al.* (2013) observed recombination followed by karyogamy in a microcyclic rust, *Puccinia horiana*, and suggested this took place after anastomoses of germ tubes produced by basidiospores. This life cycle strategy was described by Ono (2002) as the 'anastomosis of vegetative mycelia at an early stage of basidiospore infection'. This would be the most likely description of the life cycle in *A. psidii* observed in the present study. Confirmation of the presence of such anastomoses using microscopy is required to support the proposed life cycle in *A. psidii*.

Recombination has not been described in natural populations of *A. psidii*, and a study on the population in Australia determined there was no evidence of recombination since its arrival (Machado *et al.*, 2015). However, studies on populations of *A. psidii* have determined that there are different multilocus genotypes within one strain, for example the strain on *Eucalyptus* in Brazil (Graça *et al.*, 2013) and the pandemic strain on multiple hosts in the Pacific (Ross-Davis *et al.*, 2014). In the study by Graça *et al.* (2013), isolates of rust from *Eucalyptus* were either heterozygous or homozygous at two microsatellite loci, and these results were attributed to parallel mutations. Different multilocus genotypes in strains of *A. psidii* have not been discussed in light of recombination, and importantly, the results from artificial inoculations in the present study show that evidence of recombination in natural populations can be linked to infections by basidiospores.

Austropuccinia psidii is not strictly clonal, contrary to what has been hypothesized in other studies (Graça *et al.*, 2013; Morin *et al.*, 2014). This has implications for programmes that breed plants for resistance to the rust. It is also important for the maintenance of strict quarantine regulations in countries where *A. psidii* is not endemic. As has been argued, biosecurity must aim to prevent the flow of genes between organisms as much as be concerned about the identification of a pathogen (McTaggart *et al.*, 2016a).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Allelic profiles of five microsatellite markers for all scored loci. Each profile is labelled according to whether it was scored as a heterozygous or homozygous locus.

Figure S2 Nuclear state of basidiospores. Basidiospores under light microscope (a,c,e). Nuclei of basidiospores stained with DAPI under UV light (b,d,f).