



Epidemic spread of smut fungi (*Quambalaria*) by sexual reproduction in a native pathosystem

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Abstract *Quambalaria* are fungal pathogens of *Corymbia*, *Eucalyptus* and related genera of Myrtaceae. They are smut fungi (Ustilaginomycota) described from structures that resemble conidia and conidiophores. Whether these spore forms have asexual or sexual roles in life cycles of *Quambalaria* is unknown. An epidemic of *Q. pitereka* destroyed plantations of *Corymbia* in New South Wales and Queensland (Australia) in 2008. We sampled 177 individuals from three plantations of *C. variegata* and used AFLPs to test hypotheses that the epidemic was spread by asexual reproduction and dominated by a single genotype. There was high genotypic diversity across ≥ 600 AFLP loci in the pathogen populations at each plantation, and evidence of sexual reproduction based on neighbour-net analyses and rejection of linkage disequilibrium. The populations were not structured by host or location. Our data did not support a hypothesis of asexual reproduction but instead that

Q. pitereka spreads exclusively by sexual reproduction, similar to life cycles of other smut fungi. Epidemics were exacerbated by monocultures of *Corymbia* established from seed collected from a single provenance. This study showcases an example of an endemic pathogen, *Q. pitereka*, with a strictly outbreeding life cycle that has caused epidemics when susceptible hosts were planted in large monoculture plantations.

Keywords Perennial pathosystem · Obligate outcrosser · Forest pathogen · Population biology

Introduction

Host-specific pathogens must overcome continua of host resistance and spatial challenges to enable spread in native ecosystems (Barrett et al., 2009). Monocultures reduce spatial challenges of dispersal and provide temporal opportunities of synchronised susceptible host material; pathogens that use asexual reproduction have increased epidemic potential in agricultural/horticultural environments (Drenth et al., 2019). Examples of pathogens on hosts in their native range that have benefited from monocultural propagation include species of *Melampsora* (poplar rusts), *Cronartium* (pine-gall rusts) and *Dothistroma* (pine needle blight), and *Microcyclus ulei* (rubber leaf blight) (Barrès et al., 2008; Barrès et al., 2012; Drenkhan et al., 2016; Samils et al., 2021).

Quambalaria (Quambalariaceae, Microstromatales, Basidiomycota) are obligate pathogens of eucalypts

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and are thought to have originated in Australia (Roux et al., 2006). Two species, *Q. eucalypti* and *Q. pitereka*, have economic and environmental impact on *Eucalyptus* and *Corymbia*, especially when these trees are grown in plantations (Alfenas et al., 2001; Pegg et al., 2008; Roux et al., 2006). Pegg et al. (2011b) and Stone et al. (1998) showed phenotypic diversity in aggressiveness of *Q. pitereka*, and variation in the susceptibility of different provenances of *Corymbia* (*Corymbia citriodora* subsp. *citriodora* and subsp. *variegata*, *C. henryi* and *C. maculata*), to *Q. pitereka* in Australia. These continua of aggressiveness and resistance present in a native pathosystem limit epidemic potential. In contrast monocultures of *Corymbia* grown in plantation settings using seed sourced from single provenances were severely impacted by an epidemic of *Q. pitereka* that stunted and deformed trees due to repeated infection of young shoots (Pegg et al., 2008; Pegg et al., 2011a).

Species of *Quambalaria* are described from stages that resemble conidia and conidiophores (de Beer et al., 2006), and their disease and life cycles are not well understood (symptoms and morphology shown in Fig. 1). Begerow et al. (2006) hypothesised that members of Microstromatales produce basidia directly on infected tissue of woody plants, however this has not been tested for species of *Quambalaria* and it is unknown whether they reproduce clonally and/or sexually. The spores described as conidia in *Quambalaria* may

rather be gametic basidiospores, typical of other smut fungi.

A better understanding of the epidemics of *Q. pitereka* in monoculture plantations of *Corymbia* and insight into the underlying biology and genetics of this pathogen is needed. We sought to answer the following three questions: i) does clonal reproduction drive epidemics of *Quambalaria*, ii) is one dominant pathogen genotype the cause of epidemics, and iii) are epidemic populations of *Q. pitereka* sub-structured by host or location. Knowledge of the population biology of *Q. pitereka* may aid development of management strategies, including deployment of *Corymbia* resistant to disease.

Methods

Sampling

Diseased leaves and shoots were sampled from epidemics in three different plantations of *Corymbia variegata*: Beaudesert (42 trees) and Traveston (39 trees) in Queensland, and in Grafton (15 trees), New South Wales. The plantations of *C. variegata* were monocultures with a provenance from the Richmond Range (New South Wales) in Beaudesert and Traveston, and were a mix of clonal trees (propagated from

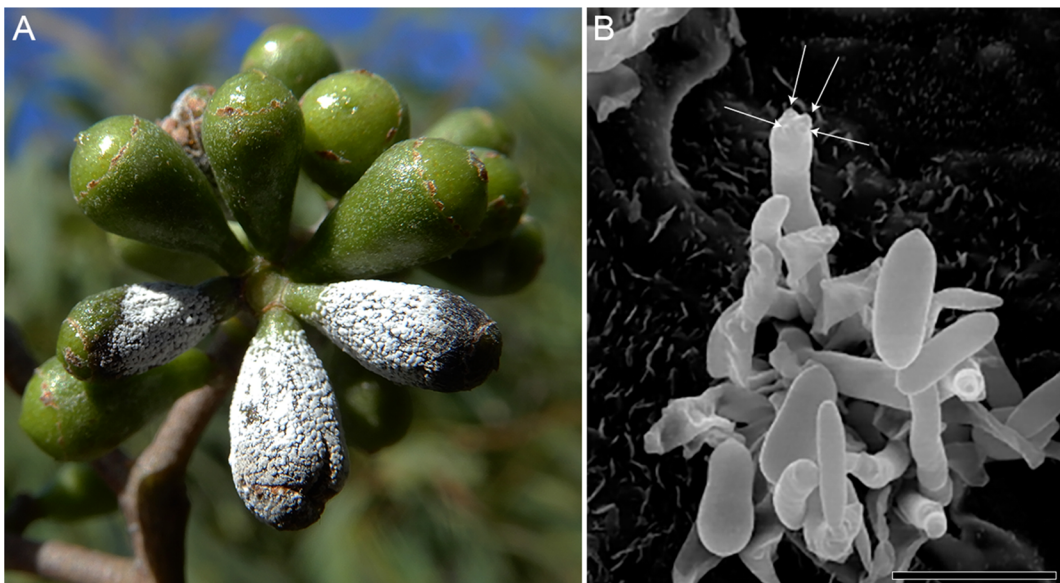


Fig. 1 A Infection caused by *Quambalaria pitereka* on fruits of *Corymbia tessallaris*. B Basidium with basidiospores and labelled sterigmata of *Q. pitereka*. Scale = 10 µm

cuttings) and trial plantings from different provenances at Grafton. We sampled stems, leaves and shoots from nearby trees of *C. citriodora* (6 trees) and *Corymbia maculata* (3 trees) outside of plantations at Grafton to test whether the epidemic was sub-structured by host.

We cultured from single leaf lesions on collected samples. We isolated from different lesions on single leaves and multiple leaves from single trees to test whether there was short-distance spread of clonal genotypes, and from different compartments in plantations to test long-distance spread of clonal genotypes. Details of the cultured individuals and their hosts are provided in Supplementary Table 1.

DNA extraction and analyses of AFLPs

High-molecular weight DNA was extracted from 40 to 80 mg of freeze-dried culture, ground in liquid nitrogen and purified with the Wizard Genomic DNA kit following the protocol for plant DNA. AFLPs were amplified using the protocol described by Vos et al. (1995), including a pre-amplification of ligated product described by Pei and Ruiz (2000). Genomic DNA was digested with two restriction enzymes, *EcoRI* and *MseI*, for 3 h at 37 °C, and adaptors were ligated to cut overhangs with T4 DNA ligase. Digested product with a 1-base-pair extension was pre-amplified from the restriction site, *EcoRI*-A/*MseI*-C. This product was diluted 1/10 and amplified using four combinations of primers with a 2-base-pair extension, *EcoRI*-AC/*MseI*-CA, *EcoRI*-AC/*MseI*-CG and *EcoRI*-AG/*MseI*-CA, *EcoRI*-AG/*MseI*-CG. Amplified fragments were called as present or absent on a CEQ888 genetic analysis system (Sciex,

USA). AFLPs were called separately for the three sampling locations.

Tests for genotypic diversity at three epidemic sites

The scored AFLPs were treated as dominant loci for analyses in GenoDive v.3.04 (Meirmans, 2020). Genotypic diversity was analysed to test whether epidemics of *Q. pitereka* were caused by one dominant genotype at each of the three sampled locations (≥ 600 AFLP loci at each site). Genotypes were assigned as clonemates using an Infinite Allele Model in GenoDive with a threshold based on the lowest number of pairs between the first and second peak, and the probability of finding the observed clonal diversity under random mating calculated using the effective number of genotypes (Gómez & Carvalho, 2001; Meirmans & Van Tienderen, 2004). Genotypic diversity was assessed using the Simpson Index and Genotypic Evenness calculated in GenoDive.

Tests for reproduction and population sub-structure across the epidemic

Data were combined from homologous AFLP loci if they could be confidently scored across the three sampled sites. Individuals were included if they were scored for 147 homologous loci. The presence of substructure within the entire sampled population of *Q. pitereka* was tested using K-means clustering of individuals from an Analysis of Molecular Variance implemented in GenoDive and assessed by the pseudo-F-statistic (Caliński & Harabasz, 1974). The clustered genotypes from the pseudo-F-statistic were visualised by

Table 1 Genotypic diversity at three epidemic locations of *Quambalaria pitereka*

Location	Number of individuals	Number of loci	Number of genotypes	Simpson Index	Genotypic Evenness	Effective number of genotypes		
						Observed	Expected	P-value*
Beaudesert	65	637	65	1.0	1.0	61.232 ^a	65	0.001
Grafton	29	600	29	1.0	1.0	25.485 ^b	29	0.001
Traveston	83	629	83	1.0	1.0	55.112 ^c	83	0.001

* Significant P value (≤ 0.001) indicates independent recombination events caused departure from expected genotypic diversity based on 999 permutations of randomised genotypes

^a Threshold for genetic distance between clones = 2

^b Threshold for genetic distance between clones = 15

^c Threshold for genetic distance between clones = 3

converting the dataset to presence/absence data and using a Principal Components Analysis in *adeget* (Jombart, 2008; R Core Team, 2014).

Putative recombination events were visualized for the three separate sites and in substructured populations from the combined dataset. AFLP data were converted to a distance matrix with *PopGenReport* (Adamack & Gruber, 2014; R Core Team, 2014) based on genetic distance described by Kosman and Leonard (2005), and displayed as a Neighbour-Net using SplitsTree4 v4.14.8 (Huson & Bryant, 2005).

The *ia* and *pair.ia* functions in *poppr* (Kamvar et al., 2014) were used to test for linkage disequilibrium in the substructured populations identified by K-means clustering, where significance for linkage disequilibrium would suggest clonal reproduction. To reduce the probability of using genetically closely linked AFLP markers, the dataset was reduced to 49 loci, selecting loci that had low *rbarD* values based on the *pair.ia* function. The standardized index of association (*rbarD*; Agapow & Burt, 2005) and its probability calculated from 999 iterations of the *ia* function was used to assess linkage disequilibrium.

Results

Genotypic diversity at three different epidemic sites

Analyses of ≥ 600 AFLP loci for separate sites indicated there was high genotypic diversity and no clonal genotypes at any of the three sites (Table 1). Tests for the effective number of genotypes showed that differences between the expected and observed number of genotypes in assigned clones were from independent recombination events (P values ≤ 0.001 , Table 1). The Simpson Indices for the three sites showed a high probability that two randomly selected samples had a different genotype. The genotypic evenness showed that sites were not dominated by a single genotype as commonly seen in clonal populations.

Reproduction and population sub-structure

K-means clustering of 126 individuals and 147 homologous AFLP loci of the combined sites supported three sub-structured populations based on the pseudo-F-statistic (Fig. 2). There was no evidence of sub-

structure based on host or location within the sampled individuals using K-means clustering (Fig. 2).

The high level of reticulation seen in the Neighbour-Net analyses of substructured populations (Fig. 3) and the three separate sites (Fig. S1) is evidence that sexual reproduction gives rise to the high levels of genotypic diversity causing epidemics. Linkage disequilibrium was rejected in substructured populations after pairwise correction of loci under linkage, with values of *rbarD* that approached 0 and supported by P values > 0.01 (Table 2), providing further evidence of sexual reproduction.

Discussion

Our data rejected hypotheses that epidemics of *Quambalaria* in plantations of *Corymbia* are fuelled by asexual reproduction and caused by a dominant genotype. Instead high levels of genotypic diversity, a lack of dominant clones and lack of strong linkage disequilibrium support the hypothesis that epidemics of *Q. pitereka* are caused by sexually reproducing populations undergoing meiotic recombination. These results show that *Quambalaria* has a similar life cycle to other species of smut fungi (Ustilaginomycotina).

Smut fungi, such as *Mycosarcoma maydis* and *Ustilago hordei*, have an asexual, haploid, saprophytic, yeast stage and become pathogenic upon dikaryotisation (Bakkeren et al., 2008). Clonal genotypes cannot persist as reproduction (or plasmogamy) is a requirement of pathogenicity in the disease cycle. The high genotypic diversity, rejection of linkage disequilibrium and network analyses support that *Quambalaria* reproduces sexually and provide new knowledge on its disease cycle. Our findings support the life cycles of Microstromatales hypothesised by Begerow et al. (2006), in which a dikaryotic hymenium produces basidia directly on host tissue.

Many fungi benefit from both sexual and asexual reproduction (Gladieux et al., 2015; Taylor et al., 2015), and epidemics are often caused by clonal stages that disperse and build inocula of successful genotypes (Drenth et al., 2019). Smut fungi are strict outbreeders and epidemics caused by pathogens, such as *Quambalaria* or *Sporisorium scitamineum* (sugar cane smut), have greatest impact when hosts are plentiful and large amounts of spores are produced.

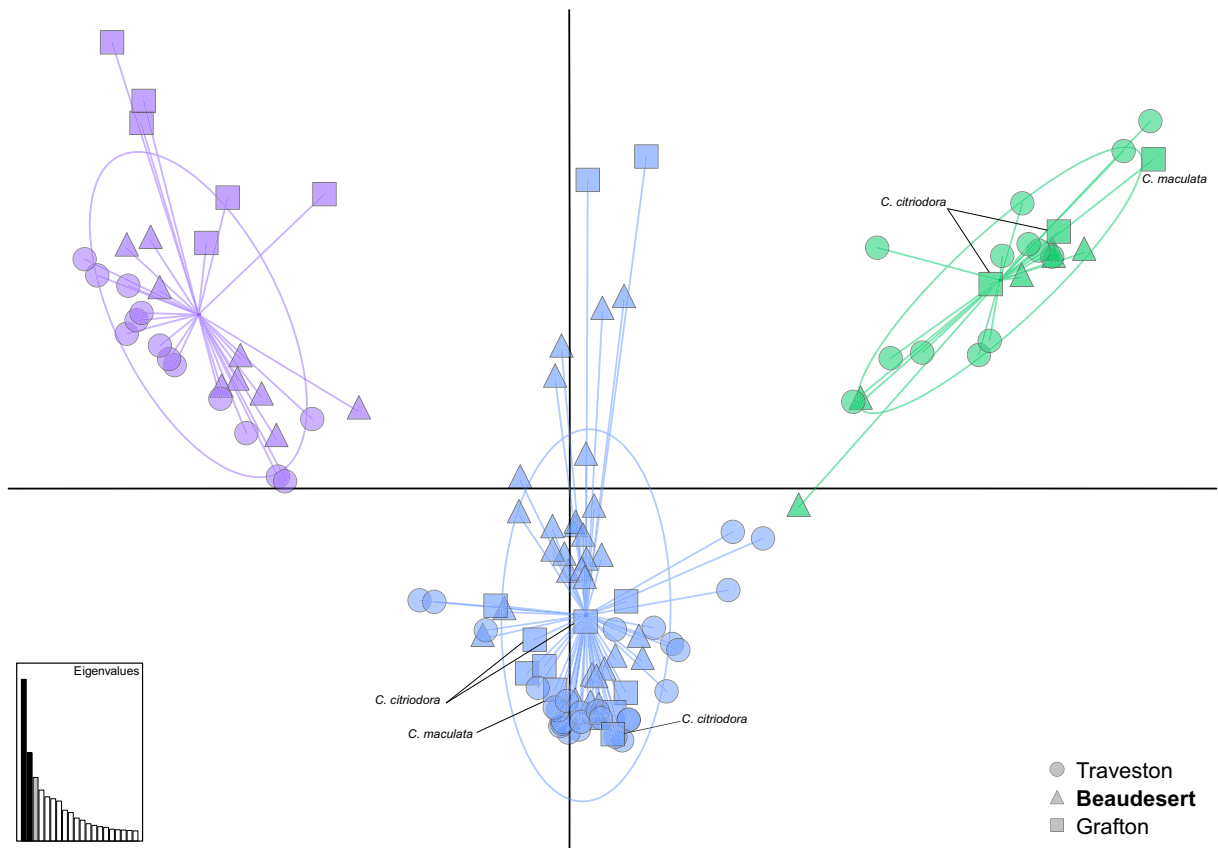


Fig. 2 A Principal components analysis of AFLP data from 147 loci constructed with *adeagenet*. Genotypes are coloured by substructure identified with the pseudo-F-statistic (3 populations),

Substructure 1 = blue, Substructure 2 = purple, Substructure 3 = green. Identified clusters are not structured by host or location. *Corymbia variegata* is the host of unlabelled genotypes

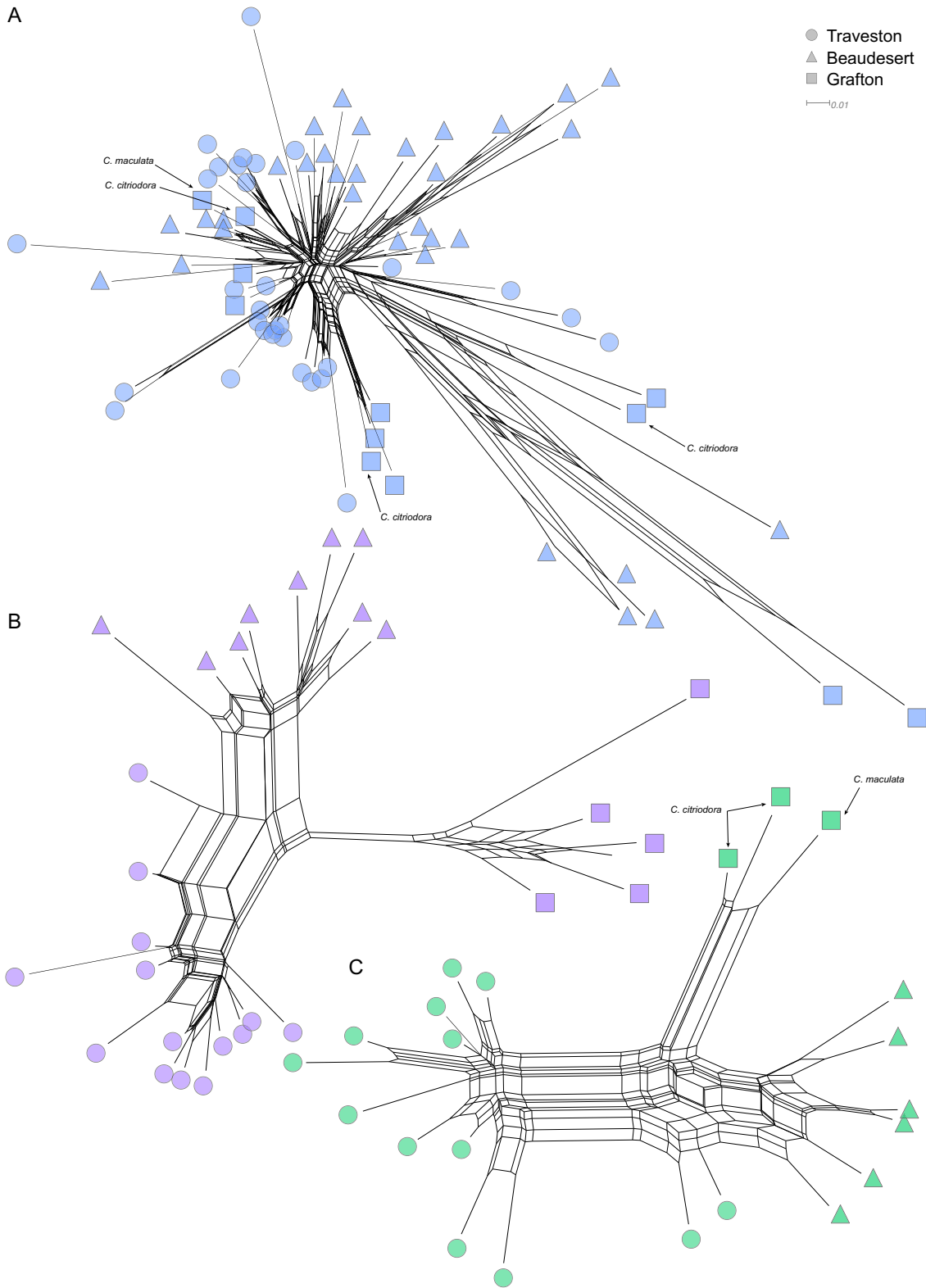
Epidemic populations of *Q. pitereka* in plantations of *Corymbia* had high genotypic diversity. It is unclear from our sampling what drove substructure in populations of *Q. pitereka*, although spatial and host structure were rejected, and long distance dispersal between sites was supported by each substructured population at each sample site. It can be hypothesized that native trees outside of

plantations bridged infections and/or were the source of infections. Planted forests of *C. variegata* in Grafton, Beaudesert and Traveston were mixes of clonal plantings, or seed sourced from the same provenance. This lack of genetic diversity with low levels of resistance in planted monocultures allowed for rapid build-up of outcrossing genotypes of *Q. pitereka*, and under

Table 2 Genotypic diversity based on substructure in populations of *Quambalaria pitereka* using combined data from three locations

Location	Number of individuals	Number of loci	Number of genotypes	Simpson Index	Genotypic Evenness	rbarD*	P value (sig. > 0.01)
Combined	126	147	140	0.99	0.98	0.02	0.04
Substructure 1	75	147	75	0.99	1.0	0.01	0.02
Substructure 2	29	147	29	0.97	1.0	0.02	0.02
Substructure 3	22	147	22	0.96	1.0	0.07	0.21

* From sites corrected by pair.ia in *poppr*



◀ **Fig. 3** Neighbour-Net analyses using SplitsTree based on Kosman genetic distance calculated from 147 AFLP loci in substructured populations: **A** Substructure 1, **B** Substructure 2, **C** Substructure 3. Reticulations indicate putative recombinant relationships between individuals. Individuals are coloured based on substructure identified by the pseudo-F-statistic and shapes indicate sample location. *Corymbia variegata* is the host of unlabelled genotypes

favourable environmental conditions resulted in disease epidemics. Self et al. (2002) and Pegg et al. (2008) reported that *Q. pitereka* is rarely found in natural, undisturbed forests. Our findings show that *Q. pitereka* is an example of a tree pathogen that spreads by recombinant spores, and has epidemic potential when conditions favour disease, such as in monoculture plantations.

A weakness of our study is the use of AFLP loci, which detect only dominant alleles and are not ideal for tests of sexual reproduction that rely on knowledge of heterozygosity (i.e. inbreeding coefficient). Our network analyses and tests of genotypic diversity showed that observed clonal genotypes, after clonemate correction among the 177 individuals sampled, were likely produced by random mating and support the hypothesis that spores produced by sexual reproduction (basidiospores) were the primary method of dispersal for *Quambalaria* in an epidemic. The variable results for the pairwise correction in the standardised index of association showed that some AFLP loci were under linkage disequilibrium, which may be explained by our close spatial sampling from the same leaf or tree, with infections caused by sister basidiospores.

Species of *Quambalaria* have low epidemic potential when they occur in diverse natural ecosystems. However, in large-scale monoculture plantations they had very high epidemic potential, to such a degree that *Quambalaria* are now considered a priority pathogen for health in plantations of *Corymbia* (Pegg et al., 2009). It is often assumed that epidemics of plant pathogenic fungi in monoculture plantations are caused by rapid, prolific and polycyclic asexual reproduction (Drenth et al., 2019). However, our results have shown that epidemic populations need to be analysed in detail to understand the reproductive biology of fungal pathogens. Insights from such studies may

aid the development and deployment of host plant resistance in efforts to reduce the impact of these diseases.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-022-02480-3>.

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Declarations

Conflict of interest The authors declare that there are no potential conflicts of interest and that the study did not involve human participants or animals. All data used in the analyses of genotypic diversity are publicly available here: https://drive.google.com/drive/folders/1Fo5DrgQnNPd8AWSumrptW_2KaGLj-Y1?usp=sharing.

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