

# Host switching between native and non-native trees in a population of the canker pathogen *Chrysosporthe cubensis* from Colombia

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The purpose of this study was to test the hypothesis that *Chrysosporthe cubensis* on native trees in South America could be the source of the pathogen that causes severe stem cankers and often mortality in commercially propagated *Eucalyptus* trees. This was done by investigating populations originating from two adjacent *Eucalyptus* (Myrtaceae) plantations in Colombia, and wild *Miconia rubiginosa* trees (Melastomataceae) growing alongside these stands. Polymorphic microsatellite markers were used to quantify allele sizes in 20 and 39 isolates from the two *Eucalyptus* stands and 32 isolates from adjacent *M. rubiginosa* trees. Gene and genotypic diversities were calculated from these data, and population differentiation and assignment tests were performed to ascertain whether the populations were genetically different. Results showed that there were no differences between any of the populations using these techniques, and that they can be treated as a single population. Therefore, the results support the hypothesis that host switching has occurred in *C. cubensis* in Colombia.

**Keywords:** *Chrysosporthe*, *Eucalyptus*, host switching, population genetics

## Introduction

Chrysosporthe canker is an economically important stem disease of plantation-grown *Eucalyptus* species in the tropics and subtropics (Wingfield, 1999, 2003). The disease is caused by three species of *Chrysosporthe* that have non-overlapping geographic ranges. These include *Chrysosporthe austroafricana* that occurs in Africa (Gryzenhout *et al.*, 2004), *C. cubensis* in South and Central America (Hodges & Reis, 1974; Hodges *et al.*, 1976, 1979; van der Merwe *et al.*, 2001, 2010) and *C. deuterocubensis* in southeast Asia (Myburg *et al.*, 2002; van der Merwe *et al.*, 2010; Pegg *et al.*, 2010). They can be differentiated by their host ranges in their native areas (Wingfield, 1999, 2003; van der Merwe *et al.*, 2010), small differences in colony morphology in culture (Gryzenhout *et al.*, 2009) and fixed housekeeping gene DNA polymorphisms (van der Merwe *et al.*, 2010).

*Chrysosporthe cubensis* has been reported from Mexico, Colombia, Venezuela, Argentina and various other countries in South America (Van Heerden *et al.*, 1997; van Zyl *et al.*, 1998; van der Merwe *et al.*, 2001; Wingfield *et al.*, 2001; Gryzenhout *et al.*, 2006, 2009). Some of the native hosts of this pathogen in South America include *Miconia rubiginosa* and *M. theaezans* (Myrtales: Myrtaceae) (Rodas *et al.*, 2005; van der Merwe *et al.*,

2010), and several *Tibouchina* spp. (Myrtales: Melastomataceae; Wingfield *et al.*, 2001). Likewise, Seixas *et al.* (2004) found *C. cubensis* on *Tibouchina granulosa* in Brazil and performed an evaluation of the susceptibility of a range of species in the Myrtales.

Previous population genetic studies on *C. cubensis* from South America have used vegetative compatibility groups (VCGs; Van Heerden *et al.*, 1997; van Zyl *et al.*, 1998). The VCG diversities of these populations were very high, and comparable to diversities observed for the closely related African species, *C. austroafricana* (Van Heerden & Wingfield, 2001). These data were later interpreted as an indication that *C. cubensis* is native to South America, and this hypothesis was supported by the occurrence of the pathogen on hosts that are native to that region (Gryzenhout *et al.*, 2009). Genetic diversities of *C. cubensis* populations from native vegetation in South America have not previously been assessed, but it would be useful to confirm that the pathogen is native to this continent. Such data would also inform efforts to breed for resistant *Eucalyptus* planting stock.

The goal of this study was to characterize the structure of *C. cubensis* populations obtained from native and non-native hosts in South America. The focus was on isolates

obtained from cankers on native *M. rubiginosa* (Melastomataceae) in Colombia (Rodas *et al.*, 2005) and from cankers on commercially propagated *Eucalyptus* trees, which were growing alongside and in close proximity to these Melastomataceae. Polymorphic microsatellite markers were employed to recover allele frequencies from *C. cubensis* populations isolated from both hosts, and population differentiation and gene flow among the hosts were tested for. The assumption was that if population subdivision was low and gene flow between the hosts was high, the probability of host switching was also high.

## Materials and methods

### *Chrysosporthe cubensis* fruiting structures, isolates and genotyping

Isolates of *C. cubensis* were obtained from two adjacent *Eucalyptus grandis* × *E. urophylla* stands on Vanessa farm, near Timba in Colombia, and from cankers on *M. rubiginosa* (a woody shrub) growing in close proximity to the *Eucalyptus* trees. On cankers from *Eucalyptus*, abundant perithecia were observed, while pycnidia were observed only on cankers where no perithecia were found. Only pycnidia were found on the cankers from *M. rubiginosa*. In total, 59 *Eucalyptus* trees were sampled from adjacent stands, i.e. 20 from stand A and 39 from stand B, while 32 *M. rubiginosa* plants occurring in and around the *Eucalyptus* stands were sampled.

Isolations were performed by sectioning through the sexual and asexual fruiting structures embedded in the bark tissue using a sterile scalpel. The pale yellow spore masses from single perithecia or pycnidia were lifted from the fruiting structures using a sterile surgical needle, and suspended in 1 mL sterile water. A 10-fold dilution was made from the original suspension, and 100  $\mu$ L was spread over the surface of 20% w/v malt extract agar (Merck) in Petri dishes. The plates were incubated at 25°C in the dark for 6–8 h, after which they were examined using a dissection microscope. Single germinating ascospores or conidia were cut from the medium using a sterile surgical needle, transferred to 20% w/v potato dextrose agar (Difco Laboratories), and allowed to grow at 25°C in the dark for 1 week.

For the purpose of population genetic analyses, a fruiting structure was sampled from individual plants, and single-ascospore or single-conidium cultures were made. This resulted in 59 single-ascospore or single-conidium isolates from *Eucalyptus* and 32 single-conidium isolates from *M. rubiginosa*. Each of these isolates was inoculated into 800  $\mu$ L 20% w/v malt extract broth (Merck) in microcentrifuge tubes and allowed to grow for 1 week in the dark at 25°C, with daily manual shaking to allow aeration. Mycelium was harvested by centrifugation, after which a modification of the CTAB (hexadecyltrimethylammonium bromide) DNA extraction protocol (Steenkamp *et al.*, 1999) was used to extract total genomic DNA from each isolate. Twelve microsatellite loci were PCR-amplified using 10 labelled primer pairs previously

developed by van der Merwe *et al.* (2010). Amplification products were subjected to genotyping using an ABI<sup>TM</sup> Prism<sup>®</sup> 3100 automated DNA sequencer (Applied Biosystems), and allele sizes for each locus were determined using the GENOTYPER computer software package.

### Population subdivision and sexual reproduction

Microsatellite loci having >50% incidence of null alleles, or being monomorphic throughout the sample of fungal isolates, were removed prior to further analyses. Additionally, all remaining null alleles were treated as missing data in population genetic analyses.

A test for population subdivision was performed using STRUCTURE v. 2.3.1 (Pritchard *et al.*, 2000). Five analyses, with priors of  $K$  ranging from  $K = 1$  to  $K = 5$  and 10 million MCMC steps, were performed to determine the optimal number of subpopulations based on the posterior likelihood values. These analyses were repeated in order to minimize the effect of the random seed number. The level of uniqueness of each subpopulation was calculated with the formula

$$\phi = \frac{\rho_S}{N_S} \cdot \frac{N_M}{\alpha_M}$$

where  $\phi$  is the level of uniqueness of the subpopulation,  $\rho_S$  is the number of private alleles in the subpopulation with  $N_S$  individuals, and  $\alpha_M$  is the total number of alleles over all loci in the metapopulation with  $N_M$  individuals. Null alleles were treated as missing data in this calculation, in order to exclude the possibility of false negative results.

Isolates were divided into three subpopulations: two from *Eucalyptus* (stand A and stand B subpopulations), and one from *M. rubiginosa*. Gene diversity ( $\bar{H}$ ) (Nei, 1973) and genotypic diversity ( $G$ ) (Stoddart & Taylor, 1988) was calculated for each of the three subpopulations. The maximum likelihood estimator of genotypic diversity ( $\hat{G}$ ) was calculated by normalizing  $G$  with the sample size. Genetic distance (Nei, 1972) between the two subpopulations from *Eucalyptus*, and the combined subpopulations from *Eucalyptus* versus the subpopulation from *M. rubiginosa* was calculated using the computer software MULTILOCUS v. 1.3b (Agapow & Burt, 2001) and expressed as Weir and Cockerham's  $\theta$  (Weir & Cockerham, 1984), which is a variation on Wright's  $F_{ST}$  (Weir & Cockerham, 1984; Agapow & Burt, 2001; Weir & Hill, 2002). This measure gives an indication of population differentiation ( $P = 0.05$ ) and ranges between 0 (low level of differentiation) and 1 (high level of differentiation). The theoretical number of migrants ( $\hat{M}$ ) was estimated from the  $\theta$  values (Slatkin, 1995) in order to yield an indication of the level of gene flow and migration between the subpopulations, and between the two hosts.

In order to test for gametic disequilibrium, i.e. the inferred level of outcrossing, the index of association ( $I_A$ ) (Agapow & Burt, 2001) for each of the three subpopulations was estimated using MULTILOCUS. The measure was estimated using one million randomizations of each of

the two data sets, normalization to yield  $\bar{r}_d$ , and comparison of the randomized distributions to the observed level of gametic disequilibrium. Randomization provides a confidence level to aid in acceptance or rejection of the null hypothesis of random mating and gametic equilibrium, while  $\bar{r}_d$  enables comparisons of gametic disequilibrium between different studies.

### Phenotypic diversity of recombinant progeny

To determine whether the sexual fruiting structures on *Eucalyptus* arose as a result of self-fertilization or outcrossing, one perithecium was sampled from each of 30 trees originating from *Eucalyptus* stand B. From each of these perithecia, 12 single-ascospore isolates were prepared as described above. These isolates were subjected to VCG and inter-simple sequence repeat PCR reaction (ISSR) assays. For VCG assays, the 12 isolates from a perithecium were paired against each other in all possible combinations, resulting in 144 pairings per perithecium. Isolates were placed on PDA-BCG plates (20% w/v potato dextrose agar, 50 mg L<sup>-1</sup> bromocresol green; Powell, 1995) in pairwise combinations, mycelium side down and approximately 3 cm apart and allowed to grow at 25°C for 10 days in the dark. Mycelial incompatibility interactions were evaluated by the identification of a dark line and yellow discoloration of the medium at the zone of contact between two isolates.

For the ISSR assays, genomic DNA was extracted from each of the 360 single-ascospore isolates as described above. These DNA samples were used as templates in ISSR reactions with primers BDB(ACA)<sub>5</sub>, DHB(CGA)<sub>5</sub> and HV(GT)<sub>8</sub> (Hantula *et al.*, 1996), in order to quantify the number of genotypes within each perithecium. Standard PCR reactions were performed with annealing at 47°C (BDB[ACA]<sub>5</sub>), 59°C (DHB[CGA]<sub>5</sub>) or 56°C (HV[GT]<sub>8</sub>), and 30 cycles (van der Merwe *et al.*, 2003). Ten microlitres of each PCR reaction was electrophoresed on a 1% w/v agarose gel and amplicons were visualized using ethidium bromide and ultraviolet light (Sambrook *et al.*, 1989).

To confirm the homothallic nature of *C. cubensis* and that self-fertilization can occur (Hodges *et al.*, 1979), a single-ascospore isolate from *Eucalyptus* stand B was artificially inoculated onto excised *Eucalyptus* twigs. Artificial wounds (5 mm<sup>2</sup>) were made with a sterile scalpel blade on five fresh twigs each of *E. grandis* and hybrid *E. grandis* × *E. camaldulensis*, respectively. The twigs were approximately 7 mm in diameter, and the bark was 2 mm thick. Twigs were surface-sterilized with 70% (v/v) ethanol, and the ends were sealed with molten candle wax. An MEA plug was cut from a fresh 3-day-old culture and placed into the wound, with the mycelium side facing the cambium. Wounds were covered with masking tape, and each twig was placed in a clean Petri dish lined with moist filter paper. Petri dishes were incubated at 25°C for 6 weeks in the dark. Once perithecia were observed, two fruiting structures were sampled and 12 single-ascospore isolates were made from each. These isolates were included in the ISSR assays.

## Results

### *Chrysosporthe cubensis* fruiting structures, isolates and genotyping

In total, 29 single-ascospore isolates were collected from *Eucalyptus* trees in stand A, 30 single-ascospore isolates from trees in the nearby stand B, and 32 single-conidium isolates from *M. rubiginosa* plants growing in close proximity to these trees. Nine of the 10 microsatellite loci (van der Merwe *et al.*, 2010) were polymorphic, and the number of alleles per locus, excluding null alleles, varied from two for locus *CcPMC* to 11 for locus *SA4*. Based on these nine polymorphic loci, 56 multilocus genotypes were identified among the isolates of *C. cubensis* collected from *Eucalyptus*. These included 20 from stand A and 37 from stand B. Isolates from stands A and B shared a single genotype. The isolates from 32 pycnidia from cankers on *M. rubiginosa* represented 26 multilocus genotypes, and none of these were coincident with those recovered from *Eucalyptus*.

### Population subdivision and sexual reproduction

STRUCTURE analyses revealed that *C. cubensis* isolates from *Eucalyptus* and *M. rubiginosa* had the same overall allelic compositions and represented a single population, i.e.  $K = 1$  (Table 1). The levels of uniqueness ( $\phi$ ) for the three subpopulations were 0.2486, 0.5304 and 0.17 for those from *M. rubiginosa* and *Eucalyptus* stands A and B, respectively (Table 2). The population from *Eucalyptus* stand A had the highest levels of genotypic diversity, uniqueness, gene diversity and private alleles (Table 2). Conversely, the population from *Eucalyptus* stand B had the lowest levels of genotypic diversity, uniqueness and

**Table 1** STRUCTURE analyses of microsatellite alleles from all included *Chrysosporthe cubensis* isolates with a range of  $K$  priors to detect population substructure. The  $K$  value, i.e. the number of subpopulations, for which the mean natural log-probability is smallest with the least amount of variance represents the most likely structure of the population

$K^a$	$\ln\Pr(X K)^b$	Mean of $\ln\Pr(X K)^c$	Variance of $\ln\Pr(X K)^d$
1	-525.1	-516.5	17.0
2	-514.9	-449.7	130.5
3	-533.3	-412.3	242.0
4	-517.3	-386.4	261.9
5	-545.5	-373.1	344.8

<sup>a</sup> $K$  represents the number of tested population subdivisions, which is used as a prior to the population subdivision analysis.

<sup>b</sup>Best natural log-probability of the data ( $X$ ), given  $K$ , during an analysis with a  $K$  prior.

<sup>c</sup>Mean natural log-probability of the data ( $X$ ), given the prior  $K$ , during an analysis.

<sup>d</sup>Variance in the natural log-probability of the data, given  $K$ , during an analysis. This value is an indication of significance, because the best-supported  $K$  prior will result in the smallest variance of the natural log-probability of the data given the prior.

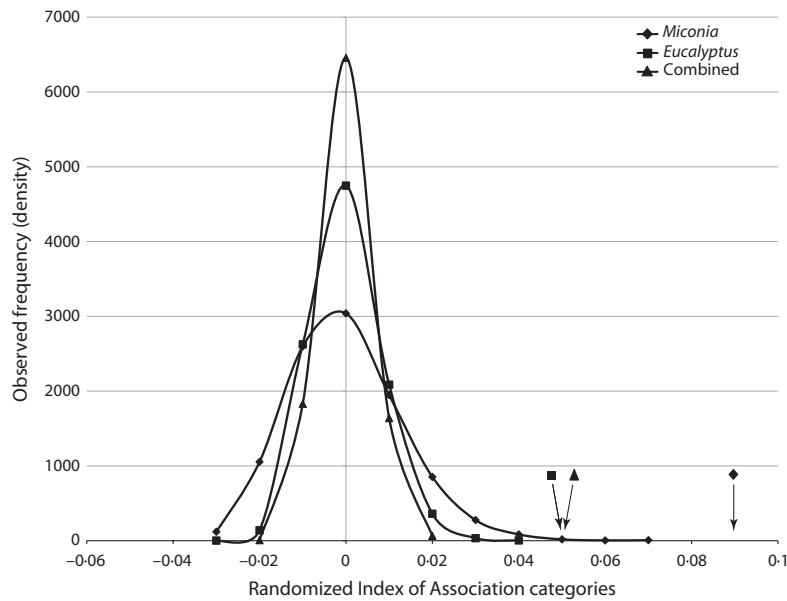
**Table 2** Population genetic statistics for the three *Chrysosporthe cubensis* subpopulations from *Miconia rubiginosa* and *Eucalyptus grandis* × *Eucalyptus urophylla* stands A and B

Statistic	<i>M. rubiginosa</i>	<i>Eucalyptus</i> stand A	<i>Eucalyptus</i> stand B
Number of isolates	32	20	39
Number of genotypes	26	20	37
Genotypic diversity, $\hat{G}$	31.37%	100%	28.47%
Number of alleles <sup>a</sup>	29	31	30
Private alleles	6	8	5
Uniqueness, $\varphi$	0.2486	0.5304	0.1700
Gene diversity	0.3983	0.4450	0.3990
Gametic equilibrium	No	No	No

<sup>a</sup>Total number of alleles over all loci for each subpopulation. The total number of alleles for the metapopulation was 46.

private alleles, while its gene diversity was comparable to that of the population from *M. rubiginosa*.

Population differentiation tests showed the two subpopulations from *Eucalyptus* to be the least differentiated ( $\theta = 0.026$ ) with the highest number of migrants ( $\hat{M} = 19.05$ ). The subpopulation from *M. rubiginosa* was more differentiated from *Eucalyptus* stand B ( $\theta = 0.194$ ;  $\hat{M} = 2.08$ ) than from stand A ( $\theta = 0.112$ ;  $\hat{M} = 3.96$ ). All three populations were in gametic disequilibrium ( $P < 0.001$ ; Fig. 1).



**Figure 1**  $\bar{r}_d$  values resulting from 10 000 randomizations of each of the *Chrysosporthe cubensis* populations from *Miconia rubiginosa* (diamonds) and *Eucalyptus* (squares) in Colombia, as well as the two populations combined (triangles). Data are the number of times (y-axis) that each of the  $\bar{r}_d$  categories (x-axis) was observed. Arrows indicate the observed  $\bar{r}_d$  values for each of the data sets ( $P < 0.0001$ ), leading to the conclusion that the null hypothesis of random association of alleles can be rejected. Therefore, the populations are in gametic disequilibrium.

## Phenotypic diversity of recombinant progeny

Among the 30 perithecia examined, 28 appeared to be the result of self-fertilization. These perithecia produced progeny representing single VCGs, while progeny representing multiple distinct VCGs were detected for only two of the perithecia (Table 3). Similarly, multiple and distinct ISSR profiles were generated for the isolates from each of these two perithecia, while those originating from the 28 other perithecia each represented single ISSR profiles. Furthermore, inoculation of a single-ascospore isolate onto twigs of *E. grandis* gave rise to perithecia that produced progeny representing a single VCG and ISSR profile (Table 3). However, no fruiting structures were observed on hybrid *E. grandis* × *E. camaldulensis* twigs after 8 weeks.

## Discussion

This study showed that *C. cubensis* is probably capable of switching between non-native *E. grandis* × *E. urophylla* and native *M. rubiginosa* in Colombia. Host switching is important in the evolution and epidemiology of plant pathogens, and is thought to occur frequently (Wingfield, 2003; Slippers *et al.*, 2005; Woolhouse *et al.*, 2005). Such host-switching events could greatly contribute to the ability of a pathogen to cause an epidemic, and are thought to be primarily associated with a change in genomic sequence at one or more loci (Woolhouse *et al.*, 2005).

Self-fertilization and outcrossing are expected to have markedly different effects on the population biology of a



**Table 3** Multilocus haplotypes and VCG phenotypes of *Chrysosporthe cubensis* isolates from sexual fruiting structures on cankers in *Eucalyptus* stand B

Perithecium number	Number of multilocus haplotypes		Average clonal fraction (Cf) <sup>a</sup>	Number of VCG phenotypes <sup>b</sup>
	Primer (ACA) <sub>5</sub>	Primer (GT) <sub>8</sub>		
Per7	6	4	75%	3
Per15	6	5	67%	6
Per1–6, 8–14,16–30 <sup>c</sup>	1	1	100%	1
Self1	1	1	100%	1
Self2	1	1	100%	1

<sup>a</sup>Cf =  $\frac{N-(C-1)}{N}$  where  $N$  is the sample size and  $C$  is the number of distinct multilocus haplotypes within each perithecium. Clonal fractions were averaged between the two primers. A clonal fraction of 100% indicates that all isolates within the sample had the same multilocus haplotype.

<sup>b</sup>For Per7, two VCGs consisted of three isolates each, while one VCG consisted of seven isolates. For Per15, three VCGs consisted of a single isolate each, one of two isolates, one of three isolates and one of four isolates.

<sup>c</sup>From 28 of the 30 perithecia, a single multilocus haplotype and a single VCG phenotype was recovered per perithecium.

fungus. This study confirmed the homothallic nature of *C. cubensis* by showing that phenotypic characters do not segregate in homothallic progeny. Tests between single-ascospore isolates from 30 perithecia collected from *Eucalyptus* revealed that only two perithecia contained more than one VCG. This result would have been expected if most of the sexual events were the result of self-fertilization in homothallic individuals, but not if outcrossing was the predominant mode of reproduction (cf. Milgroom *et al.*, 2008). However, the fact that two perithecia contained genetically different progeny provides evidence that different parental individuals were involved during fertilization. Such outcrossing events may lead to the generation of a large number of distinct genotypes. Indeed, the number of genotypes sampled from the populations was very large (Table 2), suggesting that infrequent outcrossing may be sufficient to result in a large genotypic diversity.

The clonality of the *C. cubensis* populations in Colombia supports the notion that sexual reproduction of the fungus in this region is facilitated primarily by self-fertilization. All three *C. cubensis* populations were in gametic disequilibrium ( $P < 0.0001$ ), which is a widespread phenomenon in fungi that can reproduce asexually via conidia, or have mixed mating systems (Milgroom *et al.*, 2008). Such high levels of allelic association among loci can also be linked to migration and the establishment of diversity-deficient founder populations in new areas or on new hosts (Linde *et al.*, 2009). The high levels of gametic disequilibrium and limited genotypic diversity, in lieu of non-differentiating allelic compositions in the Colombian *C. cubensis* populations, thus suggest that

only a subset of isolates were able to switch between non-native *Eucalyptus* and native *M. rubiginosa*. Therefore, movement of the fungus between populations of a host, or between different hosts, can be associated with a genetic bottleneck that may result in a decrease of genotypic diversity. However, this genotypic bottleneck, i.e. a reduction in the number of combinations of alleles, cannot be directly equated to a bottleneck in gene diversity, i.e. a reduction in the number of alleles.

A subpopulation of isolates from one of the *Eucalyptus* stands displayed greater gene diversity than the other two subpopulations, suggesting that a reduction in gene diversity may be associated with spread and host switching. Even though this may be a sampling artefact, the largest gene and genotypic diversities were nonetheless observed in a *C. cubensis* population from *Eucalyptus* stand A, while the same statistics for the other *Eucalyptus* stand and for *M. rubiginosa* were lower. These results could be interpreted in two different ways. First, they could indicate that the largest number of sexual outcrossing events of *C. cubensis* occurred in *Eucalyptus* stand A, resulting in a relatively more diverse population. This population could then have invaded stand B, as well as nearby *M. rubiginosa* plants, and this theory is supported by gene-flow statistics. The second interpretation is that the reduction in genotypic diversity was the result of an invasion taking place in a certain direction, namely from an external source which was not sampled, to *Eucalyptus* stands A and B and to *M. rubiginosa*. Such invasion-related reductions in diversity are well recognized in other invasive plant pathogens such as *Verticillium dahliae* (Atallah *et al.*, 2010) and *Cryphonectria parasitica* (Yan *et al.*, 2007; Dutech *et al.*, 2008).

The original host of *C. cubensis* in Colombia probably resides in the native Melastomataceae. Rodas *et al.* (2005) showed that a *C. cubensis* isolate from *M. rubiginosa* in Colombia was more pathogenic on *Tibouchina* spp. (Melastomataceae) than on either *M. rubiginosa* or *Eucalyptus*, while another isolate from *M. rubiginosa* was less pathogenic on *Eucalyptus* than on *M. rubiginosa*. In the current study, gene diversity values observed in all three subpopulations were comparable, and there was no statistically significant subdivision between populations from either host. If *C. cubensis* had jumped from *M. rubiginosa* to *Eucalyptus*, the gene diversity should have decreased in that direction (Woolhouse *et al.*, 2005), which is in contrast to the results obtained here. Also, a sexually reproducing ascomycete is likely to complete all stages of its life cycle on the host on which it evolved. However, sexual fruiting structures of *C. cubensis* were not observed on *M. rubiginosa* and all of the populations were in gametic disequilibrium. This suggests that both hosts (*Eucalyptus* and *M. rubiginosa*) are probably being opportunistically infected by the fungus from a yet-to-be-sampled population on a native Melastomataceae host.

The question remains as to why sexual outcrossing appears to be present in some *C. cubensis* populations, while gametic equilibrium is absent. Based on the Hardy–Weinberg equilibrium principle, one generation

of random mating can homogenize the population (Hardy, 1908). Out of 30 perithecia, only two were found that were the result of outcrossing, suggesting that mating in these populations is not random. However, if the possibility that numerous rounds of self-fertilization produced the previous generation in these populations is considered, it is likely that those genotypes would have been representative of the founder population, which was in gametic disequilibrium after a host switch. A subsequent environmental change could have initiated favourable conditions for rare outcrossing events between closely related individuals, giving rise to the sampled generations of the pathogen. Of these recombinant genotypes, some would have persisted on the original host, while others could have acquired the ability to also infect related plant species, as has been shown for other plant pathogens (De Vienne *et al.*, 2009). Therefore, occasional outcrossing appears to be an efficient strategy for *C. cubensis* to expedite the invasion of different host genotypes, or different hosts, without losing infectiousness towards the formative host.

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