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Population structure of *Holocryphia capensis* (cryphonectriaceae) from *Metrosideros angustifolia* and its pathogenicity to *Eucalyptus* species

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Abstract *Holocryphia capensis* is a recently described fungus that infects native *Metrosideros angustifolia* (Myrtaceae, Myrtales) trees in South Africa. This species is related to a number of serious canker pathogens of trees in the Cryphonectriaceae. An ability to produce lesions on *M. angustifolia* in artificial inoculation studies and its close relationship to the introduced eucalypt pathogen, *H. eucalypti*, raises concerns as to its diversity and possible impact on Myrtaceae in Southern Africa. Eight microsatellite markers were used to study the genetic diversity of *H. capensis* isolates from four sites in the Western Cape Province. All isolates could be assigned to one population, with very low gene and genotypic diversity. This was despite the fact that sexual structures of the fungus are found on infected trees. The results suggest that *H. capensis* could have been introduced into the Western Cape from a single, as yet unknown origin. Pathogenicity tests showed that *H. capensis* is pathogenic to the tested *Eucalyptus* clone.

Keywords Genetic diversity · Microsatellite markers · Myrtaceae · Pathogen spread · Tree pathogens

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Introduction

Until recently, the fungal genus *Holocryphia* (Cryphonectriaceae, Diaporthales) was monotypic and accommodated only the *Eucalyptus* (Myrtaceae, Myrtales) pathogen *Holocryphia eucalypti* (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. (Gryzenhout et al. 2006a). This fungus was originally confused with the pin oak pathogen *Endothia gyrosa* (Schwein.) De Not. (Cryphonectriaceae, Diaporthales) (Van der Westhuizen et al. 1993; Venter et al. 2001; Gryzenhout et al. 2006a) and causes superficial bark cankers on non-native *Eucalyptus* trees in South Africa, Swaziland and Uganda (Van der Westhuizen et al. 1993; Roux and Nakabonge 2010; Vermeulen et al. 2011). In Australia, *H. eucalypti* had been associated with severe stem cankers and death of plantation-grown *Eucalyptus* trees (Walker et al. 1985; Old et al. 1986; Wardlaw 1999; Carnegie 2007). Subsequent to the first description of *H. eucalypti* from *Eucalyptus* species in Australia and South Africa, it has also been reported from non-native ornamental *Tibouchina urvilleana* Cogn. (Melastomataceae, Myrtales) in Australia (Heath et al. 2007). This finding is consistent with the fact that species of Melastomataceae in the tropics, and the southern hemisphere, are commonly infected by fungi in the Cryphonectriaceae (Gryzenhout et al. 2009).

Three previously unknown *Holocryphia* species were recently described from native *Metrosideros angustifolia* (L.) Sm. (Myrtaceae, Myrtales) trees in South Africa. They included *H. capensis* S.F. Chen & Jol. Roux, *H. gleniana* S.F. Chen & Jol. Roux, and *H. mzansi* S.F. Chen & Jol. Roux (Chen et al. 2013). These species were most commonly collected from dead branches and stems of *M. angustifolia*, in the absence of clear disease symptoms on these native trees. In artificial inoculation studies, these three species all produced obvious lesions on the branches of *M. angustifolia* trees (Chen et al.

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2013). The latter study showed that isolates of *H. eucalypti*, previously reported from a *Eucalyptus* species in Swaziland, represented one of the newly described species, *H. mzansi* (Chen et al. 2013). Furthermore, it was found that *H. eucalypti* included isolates only from *Eucalyptus* in South Africa (Chen et al. 2013) and that *H. eucalypti* isolates from Australia and New Zealand represented one or more cryptic species (Chen et al. 2013). This result provided additional support for earlier studies showing that *H. eucalypti* represented a number of different cryptic species (Heath et al. 2007; Gryzenhout et al. 2010).

A recurring concern regarding fungal pathogens in the Cryphonectriaceae, particularly species of *Chrysoporthe* and *Holocryphia*, is the possibility that these fungi could expand their host ranges to other species in the Myrtales. This is supported by the occurrence of the eucalypt canker pathogen *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf. on native African *Syzygium* species (Myrtaceae, Myrtales) (Heath et al. 2006), as well as non-native ornamental *Tibouchina* species in Africa (Heath et al. 2007). In this regard, evidence suggests that *C. austroafricana* is a native African fungus that has undergone a host expansion to related, non-native *Eucalyptus* and *Tibouchina* species (Gryzenhout et al. 2009; Van der Merwe et al. 2010). Although less intensively studied, the same capacity to infect novel tree hosts in the Myrtales has been observed for species of *Holocryphia* (Heath et al. 2007; Chen et al. 2013).

There are numerous and growing numbers of examples of fungal pathogens being introduced into new environments, where they become serious pathogens of trees related to their native, and typically resistant or tolerant hosts (Wingfield et al. 2010, 2011, 2015). Well-known examples include Chestnut blight caused by *Cryphonectria parasitica* (Murrill) M.E. Barr (Anagnostakis 1987) and Dutch Elm disease caused by species of *Ophiostoma* (Brasier 2000). A more recent and highly publicised example is the rust pathogen *Puccinia psidii* Winter, a fungus native on Latin American Myrtaceae (Coutinho et al. 1998; Glen et al. 2007) that has recently entered Australia, where it is infecting large numbers of native Myrtaceae (Carnegie et al. 2010; Pegg et al. 2014).

The origin of the newly described *Holocryphia* species from *M. angustifolia* in the Western Cape Province is unknown. *Holocryphia gleniana* and *H. mzansi* were each collected from a single location and in low numbers. In contrast, *H. capensis* was found in almost all surveyed areas and it was the dominant species of Cryphonectriaceae present on *M. angustifolia* (Chen et al. 2013).

The aim of this study was to apply polymorphic microsatellite markers to investigate the population diversity and structure of *H. capensis*. Furthermore, we tested the pathogenicity of *H. capensis*, as well as *H. gleniana*, *H. mzansi* and *Diversimorbus metrosiderotis* S.F. Chen & Jol. Roux, another fungus in the Cryphonectriaceae obtained from *M. angustifolia* (Chen et al. 2013), on a commercially deployed *Eucalyptus* clone in order to understand their relative importance as pathogens of this host.

Materials and methods

Isolates

Holocryphia species used in this study were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and originated from a previous study of the Cryphonectriaceae on endemic *M. angustifolia* in South Africa (Chen et al. 2013). The isolates included 71 of *H. capensis*, four of *H. eucalypti*, three of *H. gleniana*, two of *H. mzansi* and three of *Diversimorbus metrosiderotis*.

Only *H. capensis* isolates were collected in sufficient numbers to undertake population genetic studies. Isolates of *H. capensis* came from two main geographic regions (Fig. 1), separated by at least 100 km, and each isolate was from a different tree. In Region One, samples were collected from two sites, namely Citrusdal (21 isolates) and Porterville (11 isolates), which are approximately 30 km apart. In Region Two, collections were from Stellenbosch (27 isolates) and Kleinmond (12 isolates), which are approximately 40 km apart (Fig. 1).

Population genetic comparisons

DNA extraction, simple sequence repeat (SSR) PCR and GeneScan analyses

For each of the *H. capensis* isolates, actively growing mycelium was scraped from seven-day-old cultures growing on 2 % malt extract agar (MEA, 20 g Biolab Malt Extract, 20 g Biolab Agar, 1 L water) using a sterile scalpel and transferred to 1.5 mL micro-centrifuge tubes. DNA was extracted from the mycelium using the method described by Myburg et al. (1999). To degrade RNA, samples were treated with 3 μL RNase (1 mg/mL) for 12 h at 25 °C.

Eight microsatellite loci were amplified for each of the *H. capensis* isolates using eight pairs of fluorescently labelled primers developed by Nakabonge et al. (2005) for *H. eucalypti*. Amplified DNA was separated by electrophoresis on a 2 % agarose gel, stained with GelRed (Biotium, Hayward, California, USA; 3 μL DNA amplification product with 2 μL GelRed) and visualized under UV light.

PCR amplicons were size-separated on an ABI PRISM™ 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, USA), together with the GENSCAN LIZ 500 (−250) internal size standard (Applied Biosystems, Warringon, UK). Alleles were analyzed using the software

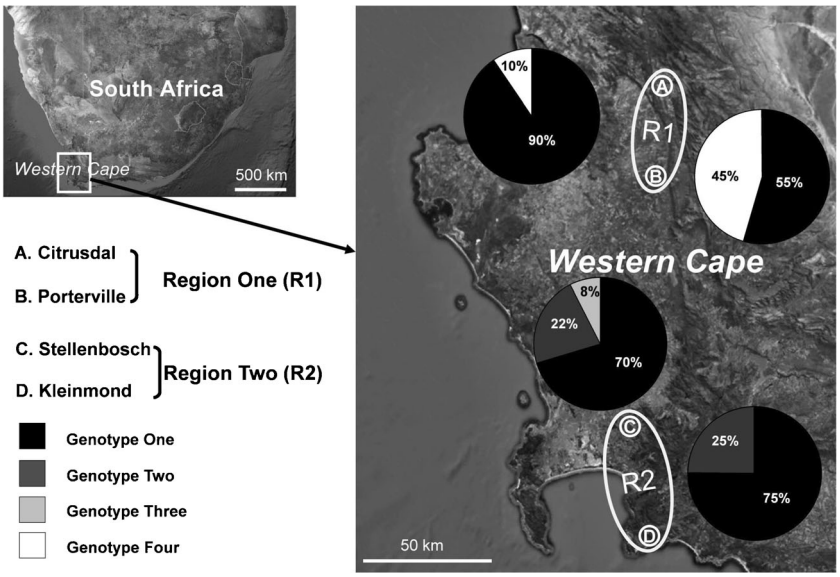

Fig. 1 Map showing the position of the sampling regions/sites in the Western Cape Province of South Africa for this study. The occurrence of four genotypes at each site is represented as percentages in the pie charts

GENEMAPPER version 3.0 (Applied Biosystems, Foster City, USA). The alleles of each locus were determined by the size of each amplicon and each allele was assigned an alphabetical character to determine the multilocus haplotype to which each isolate belonged. Isolates with the same multilocus genotype were treated as clones. Using the program POPGENE version 1.31 (Yeh et al. 1999), the total number of alleles, the number of private alleles for each locus, and the allelic frequencies were calculated.

Population assignment

Assignment of the 71 isolates into "K″ number of population(s) was achieved with the population assignment test using model-based Bayesian clustering in STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003). This method is based on the allelic frequencies of the 71 isolates, and excludes any prior geographic information. The first step was to test the optimal number of groups (K), which uses an admixture ancestry and independent allele frequency model. Twenty independent runs (100 000 steps each, with a burn-in set at 10 000 iterations) were carried out, with K ranging from 1 to 10. To determine the optimal value for K, the likelihood values of each independent run were plotted against the log-likelihood and delta likelihood values. Optimal K was determined based on higher likelihood and lower standard deviation (Evanno et al. 2005). By using the test results of K, the structure of all the isolates was evaluated in a second analysis conducted with 1 000 000 steps with a burn-in of 100 000 steps.

Population statistics

Based on the population assignment results, the gene diversity of each population was calculated in the program POPGENE version 1.31 (Yeh et al. 1999) using the equation $H = 1–\Sigma x_k^2$ (x_k is the frequency of the k^{th} allele) as determined by Nei (1973). The genotypic diversity (G) was estimated using the equation $G = 1/\Sigma p_i^2$ (p_i is the observed frequency of the i^{th} genotype in each of the populations) (Stoddart and Taylor 1988). The genotypic diversities of the two populations were compared using $\hat{G}$ (the maximum percentage of genotypic diversity), which was calculated from the equation $\hat{G} = G/N \times 100$ % (where N is the sample size; Chen et al. 1994).

Pathogenicity tests

Eight isolates of *H. capensis* (CMW37328, CMW37331–CMW37333, CMW37339–CMW37341, CMW37887), four of *H. eucalypti* (CMW7033, CMW7035, CMW7036, CMW14545), three *H. gleniana* (CMW37334–CMW37336), two *H. mzansi* (CMW37337, CMW37338), and three *D. metrosiderotis* (CMW37321–CMW37323) were selected for inoculations on a single *Eucalyptus* clone (TAG 5). This clone was chosen because previous studies have shown that it is susceptible to infection by the canker pathogen *C. austroafricana* (Van Heerden et al. 2005).

Inoculations were conducted in a glasshouse at the University of Pretoria. Twenty isolates were inoculated onto 10 trees each of clone TAG5. Trees were approximately 2 m tall, with main stem diameters of ~10 mm. Prior to inoculation, trees were allowed to acclimatize to the glasshouse conditions of 25 °C for two weeks. All fungal isolates were grown on 2 % MEA for ten days at 25 °C before inoculation. Wounds for inoculations were made with a 7-mm-diam cork borer to expose the cambium. Agar discs the same size as the wounds and overgrown with actively growing cultures of each of the test fungi were placed into the wounds, with the mycelium facing the cambium. Sterile MEA plugs were used as negative

controls. The inoculated wounds were covered with masking tape to prevent contamination and desiccation of the inoculum. Ten *Eucalyptus* trees were inoculated for each of the 20 isolates, and ten additional trees were inoculated with sterile MEA plugs to serve as negative controls. The 210 inoculated trees were randomly arranged in the same glasshouse.

The inoculation results were evaluated after six weeks by measuring the length of lesions in the cambium. Re-isolations, to fulfill Koch's postulates, were made by cutting small pieces of wood from the lesion edges and placing them on 2 % MEA at 25 °C. Re-isolations were made for four randomly selected inoculated trees for each isolate, and from all ten negative controls.

Results of the inoculations were analysed in Microsoft Excel (2010). To describe the effects of fungal isolate vs. sterile MEA on lesion length, single factor analysis of variance was used and significance among the means of lesion lengths was tested. F values with $P < 0.05$ were considered as significantly different. For each fungal isolate and the controls, the standard errors of lesion length means were calculated.

Results

Population genetic comparisons

Simple sequence repeat (SSR)-PCR and GeneScan analyses

Loci from all 71 *H. capensis* isolates used for population analyses were successfully amplified using the eight pairs of microsatellite primers. The alleles at each locus were determined for all of the isolates. For the 71 *H. capensis* isolates, three of the eight loci (Holo1B, Holo5A, Holo7A) were polymorphic (Table 1), while the remaining five loci were monomorphic.

Population statistics

Structure analyses showed that the 71 *H. capensis* isolates, collected from four sites, belonged to the same population. Therefore, all isolates represented a single population in the remaining analyses.

Twelve different alleles were amplified in the *H. capensis* population. This population had had a very low gene (0.0629) and genotypic (1.71) diversity (Table 1). Only four genotypes were identified and one genotype was dominant (Table 2).

Pathogenicity tests

Within six weeks, all isolates representing the four *Holocryphia* species and *D. metrosiderotis* produced lesions on the *Eucalyptus* plants. Lesions did not form for the control inoculations and callus tissue had grown over the wound sites.

Table 1 Allele size and diversity of the *H. capensis* population from the Western Cape Province used in this study

Locus	Allele size	Allele frequency
Holo1B	191	0.873
	193	0.127
Holo2B	202	1
Holo5A	264	0.028
	268	0.873
	272	0.099
Holo5B	345	1
Holo7A	211	0.972
	219	0.028
Holo8A	260	1
Holo9A	277	1
Holo10A	391	1
Number of isolates		71
Number of alleles		12
Gene Diversity (Nei 1973)		0.0629
Number of genotypes		4
Genotypic Diversity (Stoddart and Taylor 1988)		1.71
Percentage maximum diversity of genotype		2.41 %

With the exception of isolates CMW37887 (*H. capensis*), CMW37321 and CMW37323 (*D. metrosiderotis*), all isolates produced significantly ($P < 0.05$) longer lesions than the controls (Fig. 2). Three isolates of *H. eucalypti* (CMW7033, CMW7035 and CMW14545) were in most cases slightly more aggressive than isolates of the other three *Holocryphia* species (Fig. 2). One isolate of *D. metrosiderotis* (CMW37322) gave rise to lesions of similar size to three of the most aggressive *H. eucalypti* isolates (Fig. 2). All four *Holocryphia* species and *D. metrosiderotis* could be re-isolated from the lesions, and control inoculations were free of any of the inoculated fungi.

Discussion

Eight microsatellite markers previously developed for *Holocryphia eucalypti* (Nakabonge et al. 2005) were tested to determine the genetic diversity and population structure of *H. capensis.* Limited genetic diversity and a low number of alleles were found for the fungus. Fifty-three of the 71 isolates had the same multilocus genotype and this genotype was widely distributed throughout the sampled regions. An additional three genotypes had a limited geographic distribution. Results of structure analyses suggested that all isolates belonged to the same population. This was despite the more than 150 km geographic separation between the areas from which the isolates were collected.

Table 2 Allele size (base pairs) and number of isolates from different geographic sites for the four genotypes of *H. capensis* used in this study

Genotype No.	GenBank	Locus/Allele size								No. of isolates				
	accession no[a]	Holo1B	Holo2B	Holo5A	Holo5B	Holo7A	Holo8A	Holo9A	Holo10A	Site A	Site B	Site C	Site D	Total
Genotype 1	JQ862854	191	202	268	345	211	260	277	391	19	6	19	9	53
Genotype 2	JQ862854	193[b]	202	268	345	211	260	277	391			6	3	9
Genotype 3	JQ862861	191	202	264[c]	345	219[b]	260	277	391			2		2
Genotype 4	JQ862863	191	202	272[c]	345	211	260	277	391	2	5			7

[a] ITS sequence produced in this study for each genotype are identical to those with GenBank numbers presented

[b] Private alleles in the four genotypes

[c] Unique but non-private alleles in the four genotypes

The limited genetic diversity and lack of genetic structure in the *H. capensis* population was surprising given that sexual structures were previously reported to be common on infected branches (Chen et al. 2013). Population genetic inference also indicated that alleles in the overall sample were randomly associated, confirming that sexual outcrossing can be associated with sexual structures in this fungus. Nevertheless, two of the three polymorphic loci were in gametic disequilibrium, while five of the eight microsatellite loci were monomorphic. This suggests that either sexual reproduction is not the primary reproductive strategy, or that the isolates sampled in this study originated from a single source with a low level of diversity. Collectively, these findings could suggest a limited introduction of the fungus into a new area or a recent host range expansion (Barton and Charlesworth 1984; McDonald 1997; Nakabonge et al. 2008).

A relatively low level of polymorphism was observed for *H. capensis* isolates in this study and this could be interpreted as resulting from the applied markers having been developed for a different species of *Holocryphia*. We do not believe that this is the case given that microsatellite markers developed for population studies of one fungal species have commonly been tested and used on related species. For example, the same microsatellite markers were used for population studies of different species of *Botrytis* (Walker et al. 2011), *Chrysoporthe* (Van der Merwe et al. 2003, 2010) and *Ceratocystis* (Barnes et al. 2005; Kamgan et al. 2009). The microsatellite markers previously developed for *H. eucalypti* (= *Cryphonectria eucalypti*) have also been successfully used for population studies of isolates from South Africa and Australasia (Nakabonge et al. 2008). These primers were polymorphic for both South African and Australian populations. The isolates from South Africa and Australia were recently identified as distinct species of *Holocryphia* (Chen et al. 2013).

Analysis of the *H. capensis* isolates from four sites indicated that the population is admixed and had no sub-structure. In

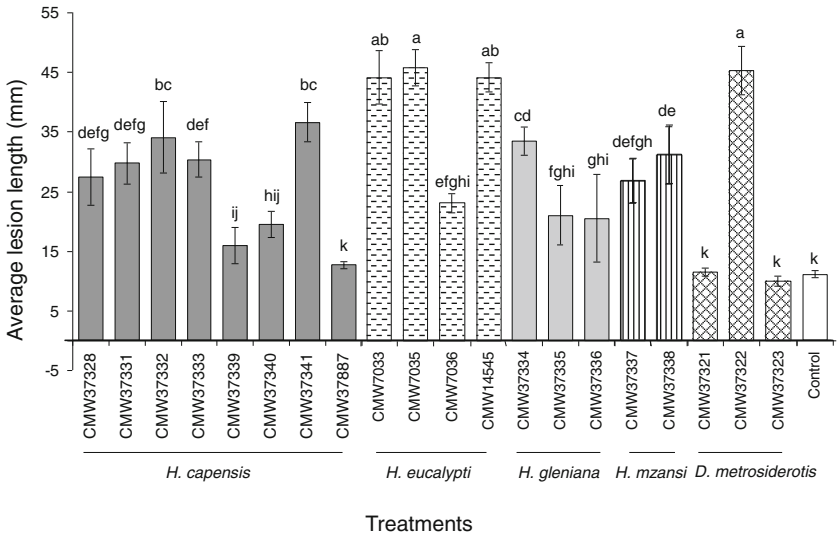

Fig. 2 Bar graph showing the average lesion length (in millimetres) resulting from inoculation trials with *H. capensis* (CMW37328, CMW37331–CMW37333, CMW37339–CMW37341 and CMW37887), *H. eucalypti* (CMW7033, CMW7035, CMW7036 and CMW14545), *H. gleniana* (CMW37334–CMW37336), *H. mzansi* (CMW37337 and CMW37338), *D. metrosiderotis* (CMW37321–CMW37323) and the controls onto an *E. grandis* clone (TAG5) under glasshouse conditions. Vertical bars represent standard errors of means. Different letters above the bars indicate treatments that were significantly different ($P = 0.05$)

addition to the presence of perithecia (sexual structures), and the detection of gametic equilibrium in some loci, these findings suggest that the fungus could be native to the African continent. However, lack of diversity, despite assessment of eight microsatellite loci, indicate that the native area of the fungus is in another, presently unsampled location. Additionally, *M. angustifolia* might not be the host on which *H. capensis* has evolved, and consequently this host probably contributes to the decay in genetic diversity of the fungus. A similar situation has been hypothesized for *Chrysoporthe cubensis* in Colombia (Van der Merwe et al. 2013).

Several of the *Eucalyptus* stem canker pathogens in the Cryphonectriaceae have been shown to occur on trees residing in other genera and species of the Myrtales. For example, *C. austroafricana*, a native African fungus, has been reported from native African *Syzygium* species (Heath et al. 2006; Nakabonge et al. 2006; Vermeulen et al. 2011) as well as non-native *Tibouchina* (Myburg et al. 2002) and *Eucalyptus* (Nakabonge et al. 2006) species. Similarly, in South America, *C. cubensis* has been isolated from native *Clidemia sericea* D. Don, *Rhynchanthera mexicana* DC. and *Miconia* spp. (Melastomataceae, Myrtales) (Rodas et al. 2005; Gryzenhout et al. 2006b), and from non-native *Eucalyptus* spp. (Gryzenhout et al. 2004) and *Lagerstroemia indica* (L.) Pers. (Lythraceae, Myrtales) (Gryzenhout et al. 2006b). Results of this study show that the *M. angustifolia* infecting fungi, *H. capensis*, *H. gleniana*, *H. mzansi* and *D. metrosiderotis* have a similar potential to cross hosts. This is clear from the fact that they are all pathogenic to *M. angustifolia* (Chen et al. 2013) and they caused disease on an artificially inoculated *E. grandis* clone in this study.

Species of the Cryphonectriaceae are increasingly emerging as a high-risk group of fungal tree pathogens and their occurrence should be monitored carefully. Results of this study have shown that *H. capensis* has the potential to cause severe disease on *M. angustifolia*, although this has yet to be observed under natural conditions. Furthermore, the detection of sexual reproduction increases the possi bility that more aggressive genotypes of the fungus could emerge in the future (McDonald and Linde 2002; McDonald 2004). The capacity of *H. capensis* to cause disease on *Eucalyptus* species was also evident. This suggests that native and non-native trees in the Myrtales should be monitored carefully for the appearance of canker disease symptoms in South Africa.

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