CHAPTER 3

GENETIC COMPARISON OF *CRYPHONECTRIA CUBENSIS* ISOLATES FROM NATIVE AND EXOTIC HOSTS IN SOUTH AFRICA

ABSTRACT

Cryphonectria cubensis is one of the most important fungal canker pathogens of *Eucalyptus* trees in tropical and sub-tropical regions of the world. Although it is best known on *Eucalyptus*, this pathogen has been recorded on three genera of trees in two families residing in the Myrtales. The fungus was first reported in South Africa in 1989, and has hindered the development of clonal propagation of *Eucalyptus* spp.in the country. Recent studies based on morphological and DNA sequence comparisons have shown that *C. cubensis* in South Africa is distinct from the fungus of the same name, occurring in other parts of the world. This has led to speculation that the South African form of *C. cubensis* represents a distinct taxon, native to this country. The recent discovery of Cryphonectria canker on native *Syzygium* spp. in South Africa supports this view. The purpose of this study was to compare the genetic structure of South African isolates of *C. cubensis* from native *Syzygium* spp. and those from exotic *Eucalyptus* and *Tibouchina* spp., using vegetative compatibility tests and polymorphic DNA (SSR) markers. Unique alleles were found for several DNA markers in each of the three groups of isolates examined. This suggests that the isolates on the different hosts could have originated from a yet unknown host. There were more unique genotypes amongst isolates from *Eucalyptus* and *Tibouchina,* than from the native *Syzygium* spp. This indicated that *C. cubensis* on *Tibouchina* and *Eucalyptus* could have originated from *Syzygium* spp. and that *C. cubensis* has been on this native host for longer than it has been on the two exotic hosts. This study provides the first clear evidence that *C. cubensis* in South Africa is native to this country or at least to the African continent.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is the causal agent of Cryphonectria canker of *Eucalyptus* trees in plantations grown in the tropics and sub -tropics (Boerboom & Maas 1970, Hodges, Geary & Cordell 1979, Sharma, Mohanan & Florence 1985a, b, Wingfield, Swart & Abear 1989), causing tree death through stem girdling. Cryphonectria canker is most common in regions with relatively high temperatures, rainfall and humidity (Hodges *et al.* 1979, Sharma *et al.* 1985a,b). In many of these areas, it is regarded as an important constraint to exotic plantation forestry (Bruner 1917, Boerboom & Maas 1970, Hodges & Reis 1974, Gibson 1980, Wingfield *et al.* 1989).

C. cubensis is best known on *Eucalyptus* spp, but has also been reported from three tree genera in the Myrtaceae and Melastomataceae. While morphologically very different, species in these two tree families are closely related, as reflected in comparisons of DNA sequence data and both reside in the Myrtales (Conti *et al.* 1997). Hosts of *C. cubensis* residing in the Myrtaceae include many *Eucalyptus* spp. (Bruner 1917, Hodges *et al.* 1979, Gibson 1980, Sharma *et al.* 1985a,b, Wingfield *et al.* 1989, Davison & Coates 1991) and *Syzygium* spp. (Hodges, Alfenas & Ferreira 1986, Chapter 2, *This dissertation*). In the Melastomataceae, hosts include species of *Tibouchina* (Wingfield *et al.* 2001, Myburg *et al.* 2002a).

Recent studies have shown differences between South African strains of *C. cubensis* and those occurring in other parts of the world. Based on comparisons of sequence data for the ITS regions of the ribosomal RNA operon, Myburg, Wingfield & Wingfield (1999) showed that isolates of *C. cubensis* from South-east Asia and South America reside in discrete clades. The South African isolates grouped together with those from South America. Because symptoms associated with the fungus on *Eucalyptus* spp. in South Africa differ from those in South America, Myburg *et al.* (1999) initiated more detailed comparisons using multiple gene trees. Results showed that the South African fungus is distinct from the fungus of the same name occurring in other parts of the world and that it probably represents a distinct species (Myburg *et al.* 2002b). It has also been suggested that *C. cubensis* would more appropriately be

accommodated in a genus other than *Cryphonectria* (Venter *et al.* 2001, Gryzenhout *et al.* 2002, Myburg *et al.* 2003).

Population genetic studies on fungal pathogens have been used to gain insight into the origin and relatedness of species (O'Donnell, Cigelnik & Nirenberg, 1998, Carbone & Kohn 2001). Various techniques have been used to determine the population structure of fungi, including the determination of phenotypic characters such as vegetative compatibility groups (VCGs) (Anagnostakis & Kranz 1987, Correll, Gordon & McCain 1992, Leslie 1993). In tree pathogens, VCGs have for example been used to study populations of *C. cubensis* (Van Heerden *et al.* 1997) and the closely related chestnut blight pathogen, *C. parasitica* (Murr.) Barr. (Anagnostakis, Hau & Kranz, 1987). Numerous molecular markers have also been used to study the population biology of plant pathogens (McDonald & McDermott 1993). In recent years, co-dominant polymorphic markers have become the preferred tools to study populations of pathogens (Taylor *et al.* 2000). These markers have the advantage of allowing the detection and characterisation of multiple alleles at a specific locus. Of these markers, microsatellites are useful because they are abundant in eukaryotic genomes (Toth, Gaspari & Jurka 2000), highly polymorphic, are easy to score and are thought to be selectively neutral (Tautz 1989). A recent study has thus used polymorphic markers to study mode of reproduction, gene flow and population differentiation in *C. cubensis* (Van der Merwe 2002).

Cryphonectria canker has recently been discovered on native *Syzygium* spp. in South Africa (Heath *et al.* 2003). This discovery and the fact that the fungus in South Africa appears to be unique, has led to the view that it might be native to this country. In this study, we consider the genetic diversity of *C. cubensis* isolates from three different hosts in South Africa.

MATERIALS AND METHODS

Isolates

South African *C. cubensis* isolates used in this study originated from three tree genera (Table 1). Isolates from *Eucalyptus* were obtained from the culture collection (CMW)

of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and were the same as those used in previous studies by Van Heerden *et al.* (1997). Isolates from the native South African trees *Syzygium cordatum* Hachst. and *S. guineense* (CD.) Willd. were the same as those used in Chapter 2 (*this dissertation*). *Tibouchina* isolates collected by Myburg *et al.* (2002) were used and supplemented with isolates collected specifically for this study from the KwaZulu-Natal Province. Techniques used to make these isolations were the same as those presented previously (Chapter 2, *This dissertation*). All isolates were obtained from single conidia, each from a different tree. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Vegetative compatibility tests

VCGs were used to estimate the genotypic diversities for the isolates from exotic *Eucalyptus*, *Tibouchina* and native *Syzygium* spp. The VCGs were assessed on oatmeal agar (Van Heerden & Wingfield 2001) (Fig. 1) and a medium described by Powell (1995) using the pH indicator, bromocresol green. Only one isolate per tree was used, since previous studies have shown that *Eucalyptus* trees are consistently infected by a single VCG (Van Heerden *et al.* 1997). Each set of isolates was initially assessed individually. VCGs previously determined for the *Eucalyptus* isolates by Van Heerden & Wingfield (2001) were used for comparative purposes. VCG's were determined for the *Syzygium* and *Tibouchina* populations using the technique described by Anagnostakis (1977). Subsequently, one representative isolate for each VCG from each of the three collections was used to compare the VCG's present on the different trees.

After the incubation period, isolates forming barrage reactions, or that had not merged, were scored as incompatible genotypes (Fig. 1). The vegetative compatible isolates merged at the point of mycelial interaction and formed confluent mycelial mats (Fig. 1). To assess the diversity of the VCGs, the number of VCGs and the frequency of isolates belonging to each VCG was determined for each of the three sets of isolates. Two different statistical parameters were used to determine the degree of diversity for these isolates. The first parameter was Stoddart & Taylor's

(1988) genotypic diversity (G). To compare diversity levels between populations, the genotypic diversity (G) was divided by the sample size (N) to obtain the maximum percentage of genotypic diversity ($\hat{G}$) (Stoddart & Taylor 1988, McDonald *et al.* 1994).

The second parameter used to consider the VCG data was the Shannon Index (SI) (Bowman *et al.* 1971, Groth & Roelfs 1986). This takes into account the frequency and evenness of the distribution of a particular phenotype. The SI was normalised to obtain H_s (normalised Shanon index) before populations of different sizes could be compared (Sheldon 1969). The H_s value was subsequently treated as an indication of phenotypic diversity based on VCGs.

Analyses using microsatellite markers

DNA isolation and amplification

Mycelium from actively growing cultures (Table 1) was inoculated into 1.5 ml microcentrifuge tubes containing 750 µl, 30% (w/v) Malt Extract Broth (Merck laboratory Supplies, Germany). DNA was isolated using a modified version (Van der Merwe 2002) of the technique of Murray & Thompson (1980).

Polymorphic loci were amplified from genomic DNA from 34 isolates from *Eucalyptus* spp., 37 isolates from *Tibouchina* spp. and 62 isolates from *Syzygium* spp., using six specific primer pairs developed by Van der Merwe, Wingfield &Wingfield (2003) (Table 2). Polymerase chain reactions (PCR) consisted of 25 ng genomic DNA, 1 mM of each dNTP (Promega, Madison, Wisconsin, U.S.A.), 0.2 µM of each locus-specific primer, 0.5 units SuperTherm *Taq* Polymerase (Southern Cross Biotechnology, South Africa), 10 × Reaction Buffer (Southern Cross Biotechnology, South Africa), 2.5 mM $MgCl_2$ (Southern Cross Biotechnology, South Africa) and 0.32 M 2-pyrrolidinone (Aldrich Chemical Company Inc., U.S.A). Sterile water was used to adjust the final volume to 12.5 µl. Amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, Inc. Foster City, California).

Conditions for the PCR consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing for 1 min at 50 °C (*COL3* and *SA1* loci) or 54 °C (*SA2*, *SA6*, *SA9* and *SA10* loci), and extension at 72 °C for 1 min. The reaction was terminated with a final extension at 68 °C for 30 min to ensure completion of the reactions. PCR products were visualised on a 2 % agarose-ethidium bromide gel using ultraviolet light. Product sizes were estimated by comparison with a 100 bp standard size marker (Promega, Madison, Wisconsin, U.S.A.).

Genescan analysis

PCR products (0.2 µl) were added to 0.4 µl loading dye (Perkin Elmer Corporation, California, USA), 0.4 µl formamide and 0.2 µl of the internal size standard GENESCAN-500 TAMRA (Perkin Elmer Corporation, California, USA). Samples were denatured at 95 °C for five min and immediately transferred to ice, followed by size fractionation using a GeneScan® PAGE gel on an ABI Prism 377 Automatic DNA Sequencer. Allele sizes were determined using GeneScan® 2.1 analysis software (Perkin Elmer Corporation) and Genotyper® 3.0 (Perkin Elmer Corporation).

Statistical analyses

Statistical analysis was performed using Multilocus 1.2 (Agapow & Burt 2001). Several analyses were performed to test for partitions and to calculate genotypic diversity, linkage disequilibrium, population differentiation and gene flow for the three sets of isolates.

In order to test for the presence of partitions within the total data set, Nei's (1973) generalised measure of G_{st} was calculated. When G_{st} approximates zero, most of the gene diversity is found within subdivisions (populations) and, therefore, population differentiation is low. Conversely, when G_{st} approaches 1, each sub-division is homogeneous and most of the variation exists between subdivisions. The G_{st} value was calculated over all isolates sampled, as well as for all combinations of subdivisions based on host origin.

Genotypic diversity, as defined by Stoddart & Taylor (1988) was modelled against the number of loci (1000 re-sampling repetitions) to produce a sigmoidal graph indicating

when the diversity present in the natural population had been adequately sampled. When the graph reaches a plateau, it indicates that sampling was sufficient. Genotypic diversity (G) defines the probability that two individuals considered at random have different genotypes (Nei 1973). In addition to genotypic diversity, the gene diversity (Nei 1973) was calculated for each locus to consider the probability of sampling two different alleles at the same locus, within the population. This statistic is equivalent to the heterozygosity value for diploid organisms. For each population, the mean of H ($\overline{H}$) was calculated to facilitate comparisons between populations.

Gametic (linkage) disequilibrium ($\bar{r}_s$) (Agapow & Burt 2001) was calculated to determine whether two individuals that are the same at a certain locus, are more likely to be the same at another locus and provides an indication of mode of reproduction. This analysis is similar to the Index of Association (I_A) and linkage disequilibrium ($\bar{r}_d$) (Agapow & Burt 2001) but is adapted to be applied to ordered alleles and can test whether alleles tending in the same direction are positively or negatively associated. Alleles are considered as integers and all are summated for each individual, after which the variance can be calculated. This value can be compared to the variance expected in the absence of gametic disequilibrium, which is the sum of the variance for each locus. Thus, the position of the observed value relative to a modelled randomly mating population, is an indication of the level of clonality or outcrossing within the population. The difference between $\bar{r}_s$, I_A and $\bar{r}_d$ is that $\bar{r}_s$ analysis the allelic values directly and the sums are over all individuals, whereas I_A and $\bar{r}_d$ computes a distance matrix and calculates the sums over all pairs of individuals.

Population differentiation (θ) is based on the number of characters shared, and different between populations. It, therefore, allows for the determination of differentiation between populations in either the allelic or genotypic distribution. It is calculated using a randomised data set with 1000 randomisations using Weir's (1996) formulation of Wright's F_{ST} for haploids. Gene flow is defined as $\frac{1}{\theta}$, the inverse of population differentiation, and is expressed as a relative value. Therefore, a low $\frac{1}{\theta}$-

value indicates low levels of gene flow, while a high value indicates high levels of gene flow. These values are supported by a probability value. Due to gene flow's inverse relationship to population differentiation (θ), a higher population differentiation value will result in a low gene flow value and *vice versa*.

RESULTS

Isolates

A total of 100 *Eucalyptus* isolates obtained from previous collections by Van Heerden & Wingfield (2001) were used for VCG analysis. These isolates originated from *E. grandis* in the KwaZulu-Natal Province (Fig. 2). Other isolates collected were those from *S. cordatum* (61) in the Northern Province, KwaZulu-Natal Province and the Mpumalanga Province and from *S. guineense* (1) in the Mpumalanga Province. A total of 37 isolates were collected from *T. granulosa* in the KwaZulu-Natal Province.

Vegetative compatibility tests

In the study conducted by Van Heerden & Wingfield (2001), 23 VCGs were identified from a collection of *Eucalyptus* isolates. The genotypic diversity for that population was 9.6 with a maximum percentage of genotypic diversity ($\hat{G}$) of 0.4 % (Table 3). The phenotypic diversity of this population, as measured with the normalised Shannon Index (H_s), was 55 %.

The *Syzygium* population resulted in 32 VCGs (Table 3) with an average of two isolates per VCG. Of the 62 isolates in this population, 21 belonged to a different VCG, with the largest VC group consisting of 10 isolates (Table 3). The VCG of the isolate from *S. guineense* was not represented among those from *S. cordatum*. The $\hat{G}$ value for this population was 26 % (Table 3), while H_s was 36 % (Table 3).

The *Tibouchina* population consisted of 10 VCGs with an average of 3.8 isolates per VCG (Table 3). Only two of the isolates resided in unique VCGs with six isolates representing the VCG-group including the largest number of isolates. This population had a $\hat{G}$ of 22 % (Table 3), and H_s of 24 %.

The test conducted to compare the three different populations showed that there were five VCGs common to both *Syzygium* and *Eucalyptus*. There was one VCG common to both *Syzygium* and *Tibouchina* and no VCGs were shared between the *Eucalyptus* and *Tibouchina* populations.

Analyses using microsatellite markers

Genescan analysis

A total of seven loci were screened using the six primer pairs available. The alleles for these loci ranged in size from 169 to 286 bp (Table 4). Primers designed for locus *SA9* targeted two independent polymorphic loci in the genome. All but one of these loci were polymorphic. Locus *SA1* was monomorphic (285 bp) for the isolates from *Syzygium*, while loci *COL3* and *SA2* could not be amplified in isolates from *Tibouchina*. The data points for *COL3* and *SA2* were thus treated as missing data in all subsequent analyses.

Statistical analyses

Based on G_{st} (Nei 1973), the total data set could be divided into partitions that correlate with the host from which isolates originated (G_{st} = 0.67). G_{st} between subdivisions based on host origin could also be calculated (Fig. 3). The G_{st} was indirectly related to the number of shared VCGs. The highest G_{st} (0.5) value was between the *Eucalyptus* and *Tibouchina* populations. These two populations also did not share VCGs.

The maximum percentage of genotypic diversity for the *Eucalyptus*, *Tibouchina* and *Syzygium* populations was 45 %, 33 % and 5 %, respectively. The corresponding gene diversity ($\overline{H}_s$) values were 0.43, 0.57 and 0.17. When genetic variation (G_{ST}) (Stoddart & Taylor 1988) was re-sampled per locus for each population, only the population from *Syzygium* did not reach a plateau, even though this population was comprised of the highest number of isolates (Fig. 4). The graphs obtained for both the *Eucalyptus* and *Tibouchina* populations reached a plateau, indicating that the maximum amount of genetic diversity had been sampled for those hosts (Fig. 4).

The observed genetic disequilibrium value ($\bar{r}_s$) for the *Syzygium* and *Eucalyptus* populations was within the ranges of the distribution for the randomised data set, with values of 0.12 (P=0.412) and 0.001 (P=0.928) respectively (Fig. 5A-B). The non-significant P-values indicate that the null hypothesis of random mating cannot be rejected. The observed $\bar{r}_s$-value for the *Tibouchina* population was not within the range of the distribution of the randomised data set but outside the set at 0.39, supported by a probability value of P<0.002 (Fig. 5C). Due to the high level of significance of this P-value, the null hypothesis can be rejected, indicating that the alleles are in gametic disequilibrium. This suggests that the primary mode of reproduction for this population is clonal.

Population differentiation was tested between all populations in all possible combinations. Of these combinations, only the test between the *Syzygium* and *Eucalyptus* populations resulted in a θ-value of 0.44 (P<0.001). The θ-values obtained from the partition test for the *Syzygium*/*Tibouchina* and the *Eucalyptus*/*Tibouchina* populations, was supported by significant P-values, resulting in $\frac{1}{\theta}$-values of 0 for both data sets and indicating low levels of gene flow. The test performed on the *Syzygium*/*Eucalyptus* populations, however, resulted in a higher $\frac{1}{\theta}$-value of 2.27. These findings correlate with gene flow estimated from G_{st}, which gives an indication of the number of migrants per generation between populations (Fig. 3).

DISCUSSION

In this study we compared the genetic structure of three populations of *C. cubensis* isolates originating from two exotic (*Eucalyptus* and *Tibouchina* spp.), and one native (*Syzygium* spp.) host in South Africa. We were able to show that the two exotic hosts have been colonised more recently than the native host, and possibly by isolates originating from the native host. We have also shown that South African *C. cubensis* isolates have a high level of genetic diversity, strongly suggesting that the fungus is native to this country.

When genetic variation (G_{ST}) was re-sampled per locus for each population of *C. cubensis*, it was shown that for both the *Eucalyptus* and *Tibouchina* populations, the maximum amount of genetic diversity had been sampled. The same was, however, not true for the population from *Syzygium*. This result is surprising as the *Syzygium* population consisted of the highest number of isolates. This population was also sampled over a greater geographic area than the *Eucalyptus* or *Tibouchina* populations, which were from plantations or ornamental trees. *Syzygium* trees that were sampled occurred abundantly over a wide area, whereas the *Tibouchina* trees were in a relatively limited area, and mostly from the town of KwaMbonambi. The *Eucalyptus* isolates were also from trees in a small area in comparison to the distribution of *Syzygium* isolates. The low diversity for *C. cubensis* from *Syzygium* spp., could be due to ecological homeostasis (Leppik 1970), which results in low infection levels. *Eucalyptus* and *Tibouchina* trees are being selected for advantageous traits such as wood quality, growth vigour, flower colour and many others. This could lead to a limited genetic base and uniform distribution of variation in the host. It is, therefore, conceivable that a *C. cubensis* epidemic is much more likely on the exotic hosts than on the native host, and can be more easily sampled.

The *C. cubensis* population from *Syzygium* had a relatively low level of mean allelic diversity ($\overline{H}_s$ = 0.17)at the investigated loci calculated from SSR data. In contrast, isolates on the exotic *Eucalyptus* and *Tibouchina* spp. displayed gene diversity ($\overline{H}_s$) as high as 0.43 in the former and 0.57 in the latter group. This might suggest that the centre of diversity for the pathogen is not the native host included in this study. Following the view that native populations generally tend to have high levels of genetic diversity (Tsutsui *et al.* 2000), it appears that the host of origin of *C. cubensis* in South Africa has not yet been found. Despite this, the high diversities obtained for the *Eucalyptus* and *Tibouchina* populations refute the notion that the fungus was introduced into the country, as originally proposed (Wingfield *et al.* 1989, Wingfield 1990).

Based on the number of unique alleles in each of the three populations of *C. cubensis* considered in this study, it appears that the founder population of the pathogen might be most closely related to the fungus on *Syzygium* spp. This is supported by the fact

that the population on *Syzygium* spp. possesses the smallest number of unique alleles when compared with those on *Eucalyptus* and *Tibouchina* spp. Isolates from *Syzygium*, therefore, have most of their genetic background in common with those populations. The population on *Eucalyptus* had the highest number of unique alleles (13). This suggests that it most likely originated from multiple colonisation events from different hosts. In contrast, the population present on *Tibouchina* possessed only five unique alleles. This could be due to the fact that *Tibouchina* is an ornamental tree in South Africa, and can be easily and cheaply reproduced by vegetative propagation. The host would, therefore, have a very uniform genetic base and would subsequently have been colonised by a limited number of *C. cubensis* genotypes due to natural selection (Brasier 1999). This is consistent with results obtained from the gametic disequilibrium tests for the various groups of isolates. Only the population of *C. cubensis* from *Tibouchina* had alleles that were not randomly associated, indicating that random mating does not occur on this host.

It is possible that outcrossing occurs in the population of *C. cubensis* from *Syzygium* spp., but that the resultant genotypes are not environmentally fit to colonise *Tibouchina* trees. This would be similar to the situation for *Microbotryum violaceum* (Pers.) G. Deml & Oberw. as described by Delmotte, Buchelli & Shykoff (1999). However, the test for gametic disequilibrium included only those isolates that are present on this host and not isolates from other populations, resulting in an increased association of alleles between loci. Another possibility is that the homothallic mating strategy of *C. cubensis* (Hodges & Reis, 1974) is governed by mating between closely related individuals, and subsequently leads to recombination between different alleles at a highly reduced frequency (Elliott 1994, Milgroom 1996).

Outcrossing was found to be present in populations of *C. cubensis* on *Eucalyptus* and *Syzygium* spp. Also, the partitions between the three investigated populations correlate strongly with the mode of reproduction. The strongest partitions were found between the isolates on *Tibouchina* spp., which are apparently clonal, and populations on *Eucalyptus* and *Syzygium* spp., that appear to be outcrossing. However, partitioning between the latter two groups of isolates was relatively weak (G_{st} = 0.3). It is, therefore, not surprising that gene flow exists between the two outcrossing populations.

The genotypic properties of the *C. cubensis* populations studied were similar to those for the phenotypic tests. Five VCGs are shared between the *Eucalyptus* and *Syzygium* populations, whereas only one VCG was shared between the *Syzygium* and *Tibouchina* isolates. The fact that no VCGs are shared between *C. cubensis* on *Eucalyptus* and *Tibouchina* suggests that the population on *Eucalyptus* did not originate from the population on *Tibouchina*.

This study provided the first comparison of genotypic and phenotypic characters for three different populations of *C. cubensis* in South Africa. It also represents the first consideration of the population biology of *C. cubensis* in South Africa, using DNA based tools. Results clearly show that, in contrast to previous hypothesis (Van Heerden & Wingfield 2001), *C. cubensis* in South Africa has a high diversity and is most likely native to the country. This is also consistent with results of recent studies showing that *C. cubensis* in South Africa is distinct from *C. cubensis* in the rest of the world (Myburg *et al.* 2002, Myburg *et al.* 2003). All three of the currently known hosts for *C. cubensis* in South Africa were represented in this study. Although we have shown that the fungus has been on *Syzygium* spp. for the longest period of time, we could not show that this is the host of origin for *C. cubensis* in South Africa. Our results, however, clearly indicate that identical genotypes of *C. cubensis* from *Syzygium*, occur on *Tibouchina* and *Eucalyptus* spp. The unique allele observed in the *Syzygium* spp. population could be a result of infection of isolates from the founder population on a yet undiscovered host. Further and more detailed surveys for *C. cubensis* on native Myrtales will be required to resolve this intriguing question. They will, however, be hampered by the fact that the fungus is difficult to find on native trees and that these trees also occur in relatively remote and unexplored areas of South Africa.

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Table 1: Isolates of *C. cubensis* used in this study

[a]Isolate Number	Host	Region
CMW 9363	*S. cordatum*	KwaZulu Natal, KwaMbonambi
CMW 9367, 9368	..	KwaZulu Natal, Duku-Duku
CMW 9364	..	KwaZulu Natal, Moba dam
CMW 9366	..	Northern Province, Tzaneen
CMW 9934 - CMW 9935	*S. cordatum*	KwaZulu Natal, KwaMbonambi
CMW 10036 - CMW 10045	..	..
CMW 10046 - CMW 10048	..	KwaZulu Natal, Sodwana
CMW 10049	..	KwaZulu Natal, KwaMbonambi
CMW 10050 - CMW 10060	..	KwaZulu Natal, Sodwana
CMW 10061 - CMW 10066	..	KwaZulu Natal, Amanzimgwenia
CMW 10067 - CMW 10069	..	KwaZulu Natal, Sodwana
CMW 10070	..	KwaZulu Natal, Munzi region
CMW 10071 - CMW 10087	..	KwaZulu Natal, Kosi bay
CMW 10191 - CMW 10194	*S. guineense*	Mpumalanga Province, Hazyview
CMW 9327 - CMW 9340	*T. granulosa*	KwaZulu Natal, KwaMbonambi
CMW 9341, 9342	..	KwaZulu Natal, Richards bay
CMW 9343 - CMW 9362	..	KwaZulu Natal, KwaMbonambi
CMW 9370 - CMW 9374	..	KwaZulu Natal, Durban
CRY 598 – CRY 601	*Eucalyptus* spp.	KwaZulu Natal, KwaMbonambi
CRY 606 – CRY 609	..	..
CRY 611- CRY 633	..	..
CRY 783- CRY 784	..	..

[a] CMW represents the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Table 2. Six polymorphic primer pairs used to study isolates of *C. cubensis*.

Primer name	Fluorescent label	Fragment size	Primer sequence (5' – 3')
Col 3	HEX (yellow)	172	F gatgaatacgaggtttcgtctc R ccgggatgaagatcagcacgag
SA 1	HEX (yellow)	320	F ggaatcaccaccactagcgtcc R gtgtctccgttaacgcagtggt
SA 2	FAM (blue)	285	F tcatgtgctcgaggaacttctg R tcttggaatgagattaagtcac
SA 6	FAM (blue)	210	F atcgacgatcaggttctggatc R tattgcggtaacccaattttcg
SA 9	TET (green)	191	F gctcgggctgccaatccttaag R cgccgagtttctcgccaccatc
	TET (green)	196	
SA 10	HEX (yellow)	183	F gccgagccatcgctttacgaag R ccgccgatgtgcttcttggacg

"F" and "R" denote forward and reverse primers respectively (Van der Merwe *et al.* 2003)

Table 3. Comparison of maximum percentage of genotypic diversity of the *Syzygium, Eucalyptus* and *Tibouchina* populations as calculated using VC tests.

					Microsatellite analysis		VCG analysis	
Population	**Collection area**	**No. of unique alleles**	**No. of Isolates**	*Outcrossing*	$\hat{G}$	$\overline{H}_s$	$\hat{G}$	H_s
Eucalyptus	narrow	13	34	Yes	45%[1]	0.43[1]	0.4%	55%
Tibouchina	narrow	5	37	No	33%[2]	0.57[2]	22%	24%
Syzygium	broad	2	62	Yes	5%[3]	0.17[3]	26%	36%

[1]Based on 100 isolates (Van Heerden *et al.* 2001)

[2]Based on 64 isolates

[3]Based on 38 isolates

Table 4. Allele ranges and number of loci amplified using the six polymorphic primers.

[a]Primer	Allele range	Number of loci
Col 3	169-173	4
SA 1	275-286	4
SA 2	204-207	4
SA 6	209-211	3
SA 9.1	181-195	3
SA 9.2	165-197	5
SA 10	176-189	7

[a] Primers developed by Van der Merwe *et al.* (2003)

Figure 1. Isolates placed in a predetermined pattern on oatmeal agar used to assess vegetative compatibility. White arrows show barrage lines found between vegetatively incompatible isolates. Vegetively compatible isolates formed a confluent lawn of mycelium as shown by the black arrows.

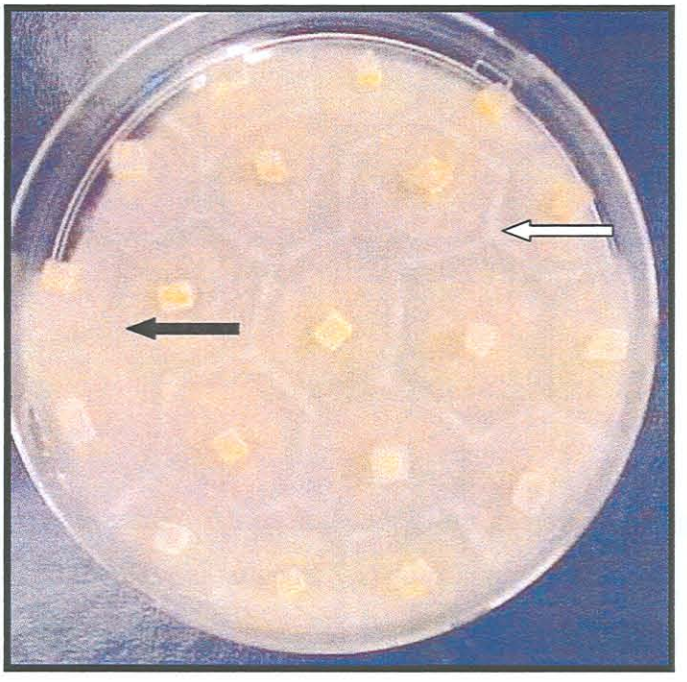
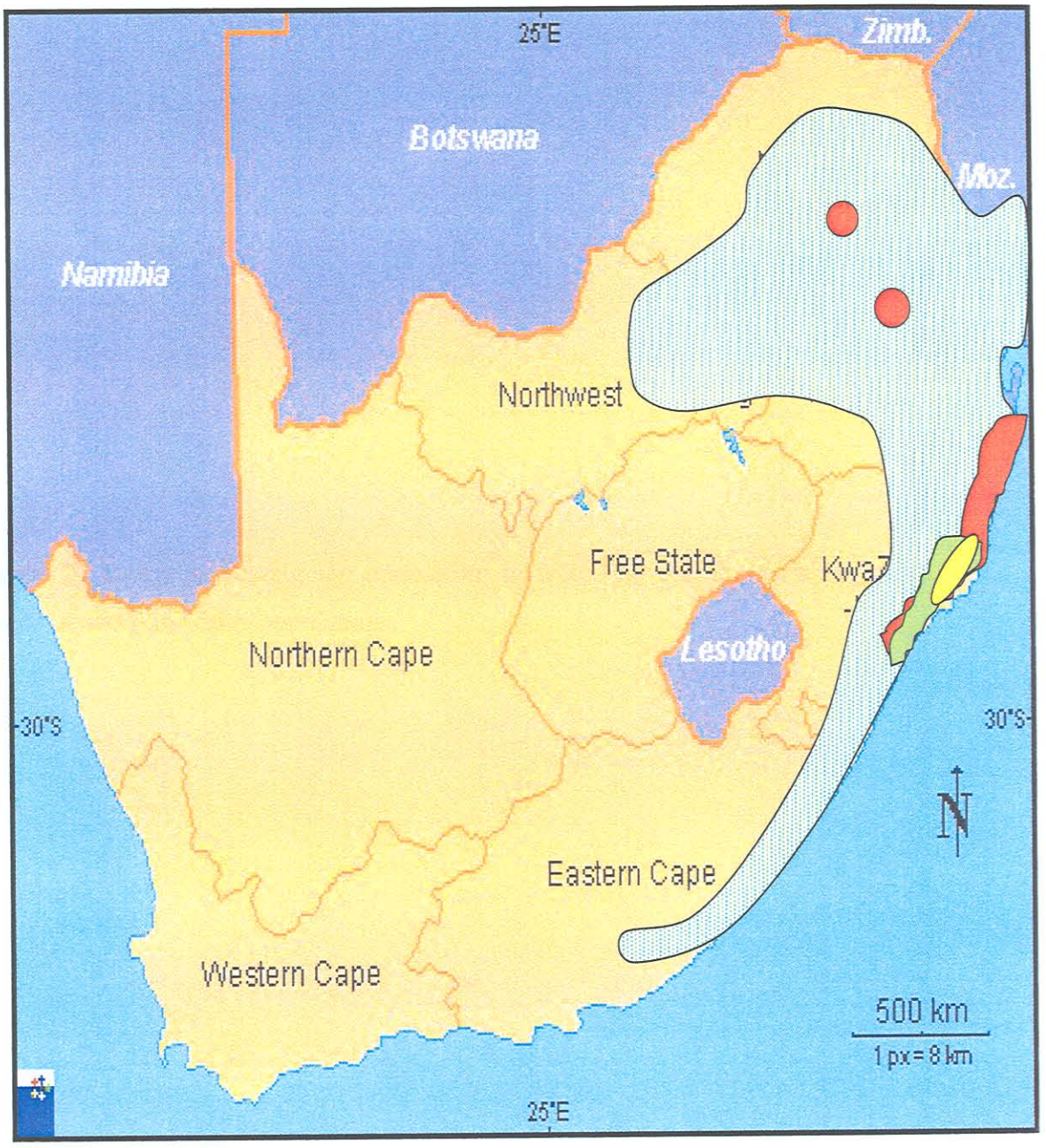


Figure 2. A map of Southern Africa indicating the geographic origin of the isolates used in the study and the natural range of *Syzygium* spp.



- Natural distributions of *Syzygium* spp.
- Isolates obtained from *Syzygium* spp.
- Isolates obtained from *Eucalyptus* spp.
- Isolates obtained from *Tibouchina urvilleana*.

Figure 3. Matrix indicating the correlation between G_{st} and the number of VCGs shared between the three populations.

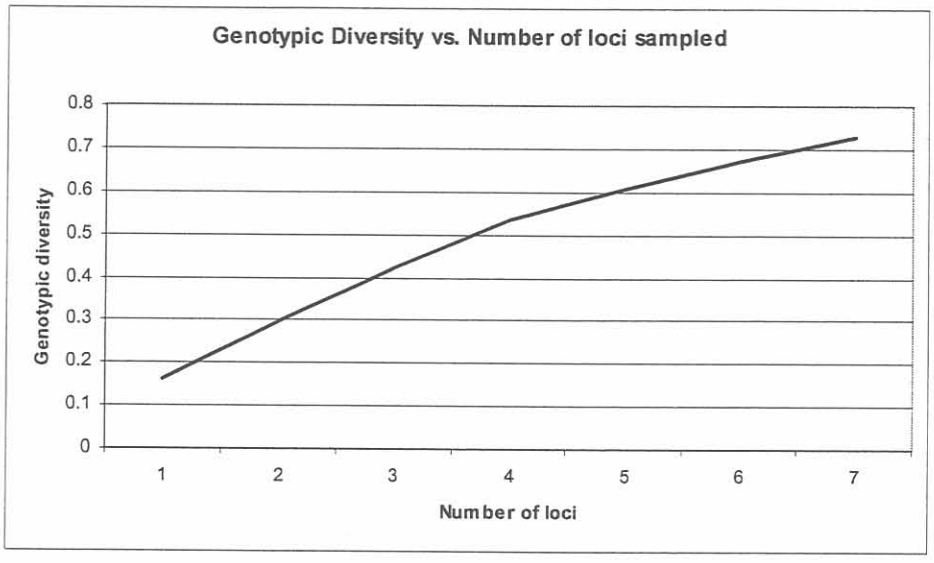
Population	*Eucalyptus*	*Tibouchina*	*Syzygium*
Eucalyptus		0	5
Tibouchina	0.5		1
Syzygium	0.3	0.4	

= G_{st}

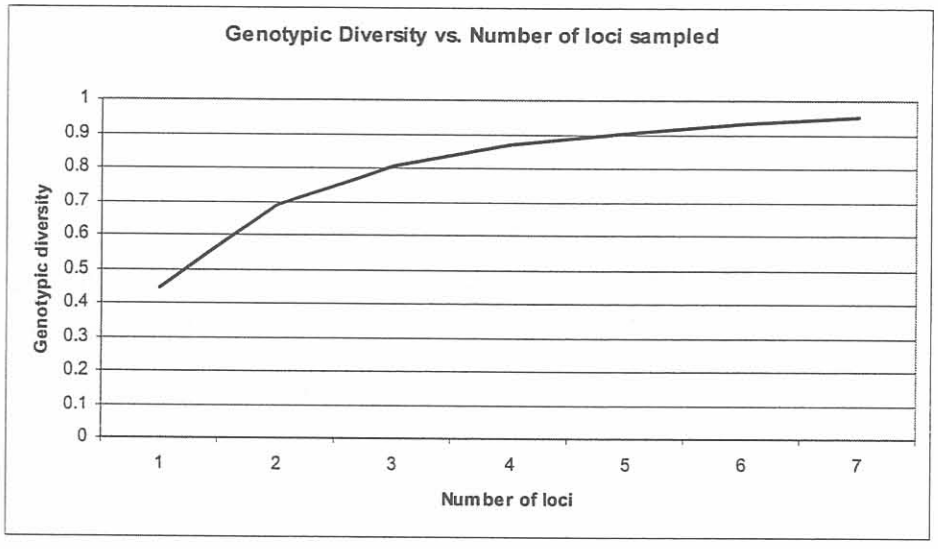
= Number of shared VCGs

Figure 4. Sigmoidal graph indicating when the diversity present in the natural population has adequately been sampled by reaching a plateau. (a) *Syzygium*, (b) *Eucalyptus* and (c) *Tibouchina* populations.

(a)


Genotypic Diversity vs. Number of loci sampled
Genotypic diversity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
Number of loci

(b)


Genotypic Diversity vs. Number of loci sampled
Genotypic diversity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
Number of loci

(c)

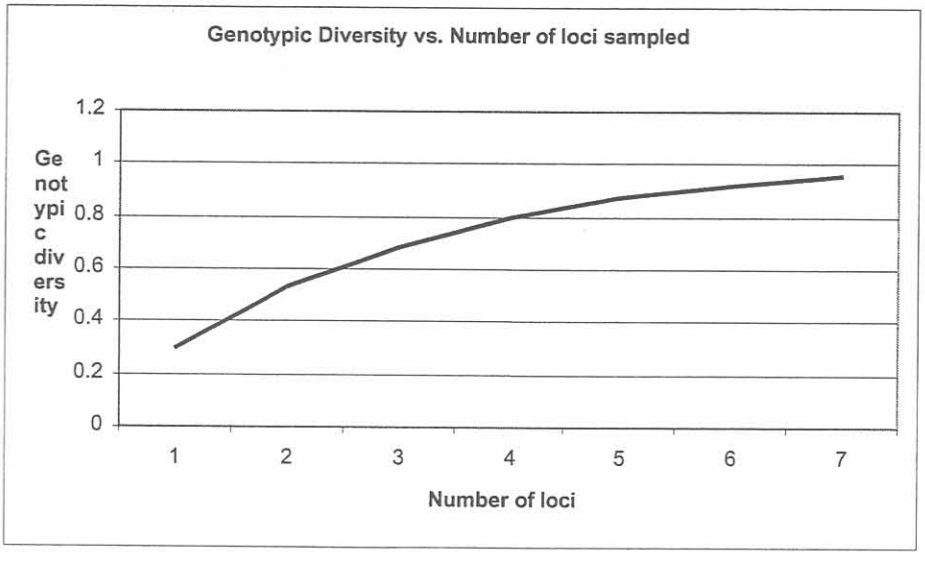
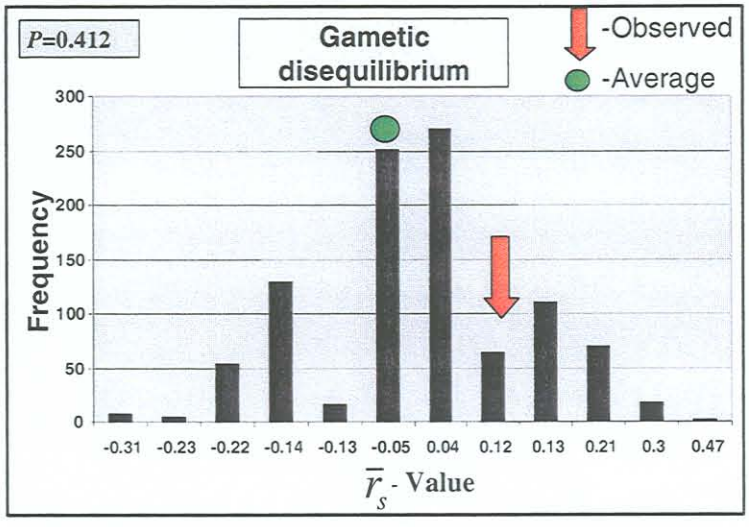
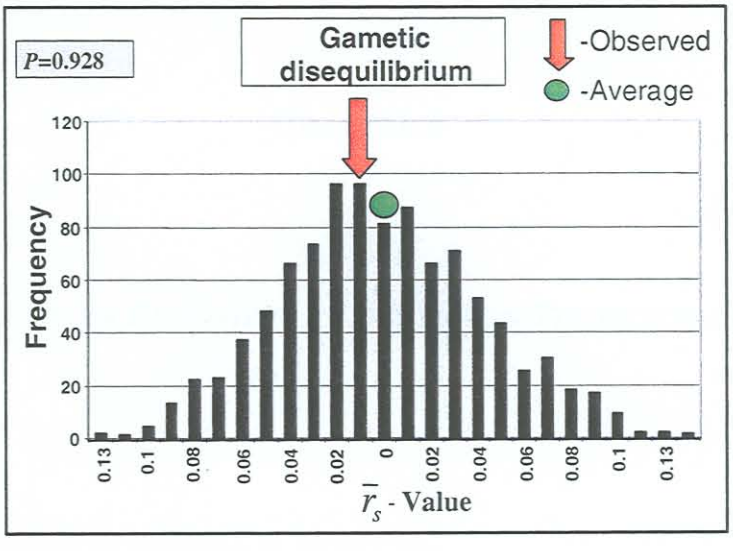

Genotypic Diversity vs. Number of loci sampled
Genotypic diversity
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Number of loci

Figure 5. Graph illustrating $\bar{r}_s$-value against allele frequency of the (a) *Syzygium* (b) *Eucalyptus* and (c) *Tibouchina* populations. The position of the observed value relative to the ranges of the distribution for the randomised data set indicates mode of reproduction.

(a)



(b)



(c)