MOLECULAR PHYLOGENY AND POPULATION BIOLOGY STUDIES ON THE *EUCALYPTUS* CANKER PATHOGEN *CRYPHONECTRIA CUBENSIS*

BY

NICOLAAS A. VAN DER MERWE

A thesis submitted in partial fulfillment of the requirements for the degree of

MAGISTER SCIENTIAE

In the Faculty of Biological and Agricultural Sciences, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria

February 2000

Study Leaders:

Prof. Brenda D. Wingfield
Prof. Michael J. Wingfield
Prof. Michael G. Milgroom

DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University

Nicolaas A. van der Merwe

February 2000

INDEX

PREFACE

Cryphonectria canker of *Eucalyptus* trees, caused by *Cryphonectria cubensis*, is a commercially important disease. This disease invariably causes yield loss due to premature death of trees or damage to eucalypt stems. In this thesis, the population diversity and taxonomy of *C. cubensis* from South Africa and Colombia are addressed.

Chapter I presents a literature review that focuses on some of the laboratory techniques used throughout the studies documented in this thesis. The review refers to molecular techniques used in population studies of fungi, as well as to studies based on vegetative incompatibility. This is a phenotypic character and represents an easy and accessible system for the determination of fungal population diversity, though it also has some disadvantages.

In Chapter II, I present a phylogenetic study of *C. cubensis* isolates from Colombia. The study was performed in order to confirm that the fungus collected from that country represents *C. cubensis*, and also to determine its relatedness to isolates from other countries.

In Chapter III, I present the results of a study to determine the mating system of Colombian *C. cubensis* isolates. This was achieved through artificial inoculations onto *Eucalyptus* twigs and assessment of ascospore progeny that was collected. It was found, through the use of vegetative compatibility groups (VCGs) and DNA fingerprinting of progeny from naturally occurring perithecia, that outcrossing occurs in the Colombian population of *C. cubensis*. This is the first report of outcrossing in *C.*

cubensis, since it was thought to be exclusively homothallic, and reproducing through self-fertilisation.

The genotypic diversity of the Colombian population of *C. cubensis* was investigated in Chapter IV. VCGs and for the first time, randomly amplified polymorphic DNA (RAPD) tests, were used to estimate the frequencies of discrete genotypes in the population. These data were statistically analysed to determine the genotypic diversity of the population.

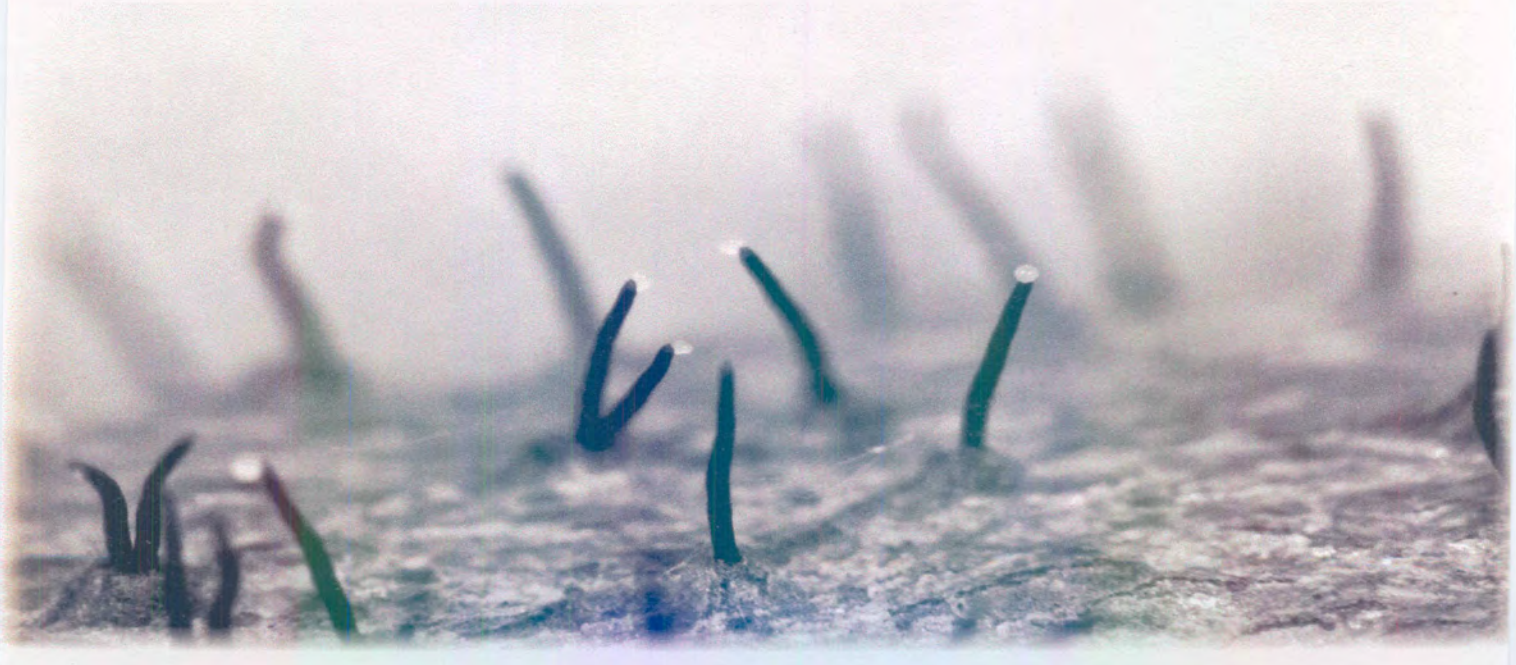
In Chapter V, I describe a novel technique for the isolation of microsatellite-like polymorphic DNA markers in fungi, and demonstrate a potential use for these markers in the taxonomy of *C. cubensis*. Using several of these markers, it was possible to discriminate between isolates of *C. cubensis* from South Africa and isolates from South America and South East Asia.

Chapter VI represents the first study of population diversity of *C. cubensis* from South Africa, at the DNA level. The population has previously been analysed for diversity using VCGs. However, VCG data did not provide a sufficiently high resolution to answer questions relating to the reproductive strategy of the population. Using polymorphic markers (developed during the study described in Chapter V), it was possible to infer that outcrossing and subsequent recombination of genes, occurs in the South African *C. cubensis* population.

CHAPTER I

VEGETATIVE INCOMPATIBILITY AND MOLECULAR POPULATION GENETICS OF FUNGI, WITH PARTICULAR REFERENCE TO *CRYPHONECTRIA CUBENSIS*

(LITERATURE REVIEW)



VEGETATIVE INCOMPATIBILITY AND MOLECULAR POPULATION GENETICS OF FUNGI, WITH PARTICULAR REFERENCE TO *CRYPHONECTRIA CUBENSIS*

INTRODUCTION

The South African forestry industry is based entirely on the planting of exotic tree species that yield a high timber quality and volume per tree (Department of Water Affairs and Forestry, 1998). Of the tree species planted, *Pinus* spp. and *Eucalyptus* spp. are the most important. Several breeding programmes to improve the quality and disease resistance of *Pinus* and *Eucalyptus* are currently operational. To optimise the breeding process, these programmes depend on knowledge pertaining to the diversity of pathogen populations.

One of the most important diseases of *Eucalyptus* in South Africa is Cryphonectria canker, caused by *Cryphonectria cubensis* (Wingfield *et al.*, 1989). Information on the population diversity of *C. cubensis* in South Africa has resulted in the development of potentially resistant *Eucalyptus* clones (Van Heerden, 1999; Wingfield, 1990). A stand of these clones was recently screened for resistance to isolates of *C. cubensis* (Van Heerden, 1999). These isolates represent the most virulent vegetative compatibility groups (VCGs), which have been identified during

studies of the diversity of populations of these fungi. Knowledge of the diversity of *C. cubensis* populations in South Africa is, therefore, crucial to the forestry industry.

In this review, I will attempt to illustrate the techniques that are used to study fungal population diversity. These are specifically related to *C. cubensis*, where possible. However, very little knowledge is available on *C. cubensis*, in comparison to other extensively studied fungi, such as the closely related *Cryphonectria parasitica*. Therefore, in this document most of the examples used pertain to fungi other than *C. cubensis*.

MOLECULAR POPULATION GENETICS

Several techniques are available to study populations of fungi at the molecular level. The advantage of DNA-based data is that it is not influenced by environmental factors, and is subsequently selectively neutral (Hartl & Clark, 1989; McDonald & McDermott, 1993). In contrast, protein electrophoresis patterns can be influenced by the physiological state of the individual at the time of protein extraction. Such analyses are, therefore, restricted to a few enzymes that are normally selectively neutral (Rafalski *et al.*, 1996).

Isozymes

Isozyme analyses have been extensively used for the identification of individuals, as well as for population studies of plants (Chan & Sun, 1997), animals (Anderson *et al.*, 1979; Puterka *et al.*, 1993) and fungi (Burdon & Roelfs, 1985a; Burdon & Roelfs,

1985b). In *C. cubensis*, isozymes have been used in the identification of isolates that differ in their levels of virulence (Alfenas *et al.*, 1984), as well as taxonomic characters in the identification of *C. cubensis* from the roots of *Eucalyptus* in Australia (Davison & Coates, 1991). The assessment of isozyme markers is generally difficult and time consuming, and the data produced have a low information content (Rafalski *et al.*, 1996). Researchers have consequently adopted DNA-based techniques for population analyses of fungi.

DNA fingerprinting

In 1985, Jeffreys *et al.* produced the first individual-specific DNA fingerprints from humans. The process involved enzymatic digestion of genomic DNA, followed by Southern hybridisation with a cloned minisatellite DNA sequence. Subsequent to this discovery, the application of DNA fingerprinting technology became of increasing importance in studies of plant pathogen populations (Bonfante *et al.*, 1997; Chen *et al.*, 1994; Goodwin *et al.*, 1992; Harrington & Rizzo, 1999; McDonald and McDermott, 1993).

In more recent years, the polymerase chain reaction (PCR) has made it possible to use arbitrary primers to amplify random portions of the genome. Techniques based on this technology include randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), randomly amplified microsatellite sequences (RAMS) (Hantula *et al.*, 1996) and amplified fragment length polymorphisms (AFLPs) (Hill *et al.*, 1996). All of these techniques have been used in fungi for the identification of strains (Baleiras Couto *et al.*, 1996), as taxonomic characters (Baleiras Couto *et al.*, 1996; Meyer *et al.*,

1999), and the elucidation of population parameters such as genetic diversity (Hantula *et al.*, 1996), sexual outcrossing rates (Milgroom *et al.*, 1993) and migration patterns (Milgroom *et al.*, 1992).

As an example, RAPD analysis has been used to detect homothallic outcrossing in *Phytophthora sojae* (Whisson *et al.*, 1994) and *Pythium ultimum* (Francis & St Clair, 1993), and also allowed a global study of isolates of *Cryptococcus neoformans* (Meyer *et al.*, 1999). The RAPD technique has recently been enhanced by the detection of microsatellite motifs within RAPD profiles (Davis *et al.*, 1997). Such advancements, together with the ease of use of this technique, makes it ideal for use in fungal population genetics, since large numbers of samples can be analysed concurrently. The disadvantages of RAPDs include problems with reproducibility and the fact that the loci detected are dominant (Williams *et al.*, 1990).

RFLPs

In *C. parasitica*, specific restriction fragment length polymorphism (RFLP) markers have been useful. These markers made it possible to conduct studies of population diversity (Milgroom, 1995), structure (Milgroom & Lipari, 1995), random mating (Milgroom *et al.*, 1993), and a comparison of genetic diversity of populations from different geographic regions (Milgroom *et al.*, 1992). The advantage of RFLP markers is that they are co-dominant, allowing for the differentiation of alleles at each locus. This attribute makes it possible to estimate outcrossing rates (Milgroom *et al.*, 1993) and to determine the relative contributions of asexual and sexual reproduction in populations (Chen & McDonald, 1996; McDonald *et al.*, 1994).

Microsatellites

Microsatellites represent an abundant source of polymorphic markers in all organisms (Groppe *et al.*, 1995; Jarne & Lagoda, 1996; Lagerkrantz *et al.*, 1993; Panaud *et al.*, 1995; Struss & Plieske, 1998). These regions consist of highly repetitive DNA sequences of two to five bases that occur throughout the genome (Lagerkrantz *et al.*, 1993; Panaud *et al.*, 1995). The source of polymorphism has been identified as unequal crossing over and to a lesser extent, polymerase slippage, during meiosis (Geistlinger *et al.*, 1997; Grist *et al.*, 1993). These characteristics provide useful information during statistical analyses of data, since formulae can be adjusted to coincide with the mutational mechanisms of microsatellites (Goldstein *et al.*, 1995; Slatkin, 1995).

In fungi, microsatellites have been used for studies of the ecology and diversity of grass endophytes (Groppe *et al.*, 1995; Moon *et al.*, 1999), and for studying host and pathogen population variation in natural plant-pathogen systems (Delmotte *et al.*, 1999). Microsatellite data also allowed separation of strains of the ectomycorrhizal fungus, *Suillus collinatus*, that possess different symbiotic capabilities (Bonfante *et al.*, 1997). It is clear that microsatellites can be applied to answer very complex questions pertaining to the biology of fungi. Other techniques such as RAPD, RFLP and phenotypic characters might not provide sufficient data to resolve these questions. However, these techniques are useful in resolving specific questions pertaining to fungal biology, and in many cases provide a simpler and cheaper alternative to microsatellite analyses.

VEGETATIVE INCOMPATIBILITY

The basis of vegetative incompatibility

All filamentous fungi possess self and non-self recognition systems that determine processes such as sexual reproduction and hyphal fusion. Vegetative incompatibility is a hyphal fusion recognition system, by which strains that belong to the same vegetative compatibility group can undergo hyphal anastomosis (Adams *et al.*, 1987). When two strains are in different vegetative compatibility groups (VCGs), *i.e.* they are vegetatively incompatible, an antagonistic response is elicited that prevents anastomosis from occurring (Newhouse & MacDonald, 1991) and, in most cases, results in the formation of barrage lines in culture (Glass & Kuldau, 1992).

Newhouse & MacDonald (1991), using transmission electron microscopy of vegetatively compatible strains of *C. parasitica*, demonstrated that hyphal anastomosis involves a complete cytoplasmic continuity, with microtubules and mitochondria extending through the fusion point of both strains. In contrast, vegetatively incompatible strains of this fungus did not undergo hyphal anastomosis, but displayed cell death involving cellular collapse and cytoplasmic degeneration at the point of contact. Anagnostakis (1977) also noted that a barrage reaction in *C. parasitica* is followed by the production of pycnidia (asexual fruiting structures) by both strains along the line of contact. Such a reaction might be due to chemical processes that result from cellular degeneration, but this remains to be investigated.

Molecular aspects of vegetative incompatibility

Vegetative incompatibility is controlled by varying numbers of heterokaryon incompatibility (*het*) loci and alleles in different fungal species. In *C. parasitica*, it was postulated that vegetative incompatibility is controlled by at least five loci (Anagnostakis, 1982b), while *N. crassa* and *P. anserina* possess 10 and 17 *het* loci, respectively (Beisson-Schecroun, 1962; Bernet, 1965; Perkins *et al.*, 1982). The large number of loci, and also the possibility of multiple alleles at a locus (Glass & Kuldau, 1992), makes it difficult to characterise biochemical pathways governing the system. Nonetheless, the genetics of vegetative compatibility has been investigated in several important fungal species.

Both allelic and non-allelic systems of vegetative incompatibility exist. In an allelic system, vegetative incompatibility is controlled by alternate alleles at several *het* loci in a haploid genome (Glass & Kuldau, 1992). In a non-allelic system, the existence of genetic differences at two separate loci results in vegetative incompatibility. Therefore, a common feature of the two systems is that incompatibility results from a genetic difference that exists between two strains. Cloned genes involved in vegetative incompatibility resulting from both systems, revealed that the alternating alleles are not idiomorphic in nature (Glass & Kuldau, 1992). Thus, they represent true alleles and not divergent sequences as found at the mating-type loci (Turgeon *et al.*, 1993).

Ophiostoma (*Ceratocystis*) *ulmi* displays vegetative incompatibility reactions of varying intensities (Brasier, 1984). This phenomenon has been ascribed to differing

numbers of incompatibility gene differences at the *het* loci of two incompatible strains, and is probably the result of sexual recombination. Sexual reproduction between two strains belonging to different vegetative compatibility groups (VCGs), can lead to recombination of alleles at the *het* loci, yielding progeny that are diverse in the number of differences they possess at these loci. These progeny will consequently represent "intermediate forms" between well-defined VCGs (Cortesi *et al.*, 1996).

The use of VCGs in population biology

Vegetative incompatibility between two strains of a fungal species can be tested at the phenotypic level (Anagnostakis, 1982b; Leslie, 1993), and thus represents an easy and simple process to gain knowledge of population diversity and structure. For this purpose, VCGs have been applied to populations of many important plant pathogens, including *C. parasitica* (Anagnostakis *et al.*, 1986; Anagnostakis & Kranz, 1987), *C. cubensis* (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998; Wingfield *et al.*, 1997), *Fusarium* spp. (Correll *et al.*, 1992), and *Aspergillus flavus* (Bayman & Cotty, 1991).

In a recent study (Bayman & Cotty, 1991), it was possible to monitor the VCG diversity of *A. flavus* infecting a single cotton field over a period of three years. The VCG diversity in this population was surprisingly high, given that *A. flavus* is an asexual fungus. The authors hypothesised that the origin of VCG diversity might be the influx of spores from nearby sites, causing changes in the relative numbers of unique genotypes. It is known that in some fungi, genetic relatedness of strains can be measured using VCGs (Puhalla & Mayfield, 1974), and these data also correlate with

the host range and virulence of strains. However, careful consideration is needed when such data are extrapolated to other fungi.

The *C. cubensis* population in South Africa is represented by several VCGs, and this character has also been used to determine its diversity (Van Heerden, 1999). *Cryphonectria cubensis* in South Africa is believed to have been introduced into the country fairly recently (Myburg, 1997; Wingfield *et al.*, 1989). If the possibility of continuous introduction can be eliminated, then it can be assumed that the diversity of the population would be nearly clonal, since sexual reproduction is very rare in this population (Wingfield *et al.*, 1997). The large number of VCGs (23 VCGs from 100 isolates) present in the South African population of *C. cubensis*, suggests otherwise. The source of variation must thus be identified in order to permit the implementation of control strategies, such as biological control through hypovirulence.

The effect of VCG diversity on biological control

Biological control of fungal pathogens through the use of virulence attenuating hypoviruses (Heiniger & Rigling, 1994; Scibilia & Shain, 1989) has become an exciting prospect. These viruses have a double-stranded RNA (dsRNA) genome (Day *et al.*, 1977; Rae *et al.*, 1989), and are present in the cytoplasm of the fungal host (Fahima *et al.*, 1993; Newhouse *et al.*, 1990). Phenotypic changes associated with the presence of hypovirulence-associated dsRNA in *C. parasitica* include reduction in sporulation capability, altered colony morphology, reduced pigmentation, and reduced laccase and cellulase activity (Anagnostakis, 1982a; Anagnostakis, 1984; Elliston, 1985; Hillman *et al.*, 1990; Rigling & Van Alfen, 1991). In *C. cubensis*,

hypovirulence is also characterised by a reduction in growth rate and discoloration of fungal mycelium from pale yellow to bright orange (Van Heerden, 1999).

DsRNA hypoviruses are transferred between strains of the same VCG when hyphal anastomosis occurs (Day *et al.*, 1977; Newhouse & MacDonald, 1991; Newhouse *et al.*, 1990). When strains are vegetatively incompatible, transfer of dsRNA is prevented (Day *et al.*, 1977). Van Heerden (Van Heerden, 1999) demonstrated that dsRNA elements can be transferred between strains of *C. cubensis* belonging to different VCGs. This is not surprising, given that Liu & Milgroom (1996) showed that hypovirus transmission between vegetatively incompatible strains of *C. parasitica* depends strongly on the number of genetic differences at the *het* loci.

Biological control of chestnut blight through the spread of hypovirulent strains has been successful in Europe, where the genetic diversity of *C. parasitica* is low (Heiniger & Rigling, 1994). In contrast, the genetic diversity of populations of the fungus in America is relatively high (Milgroom, 1995). Hypovirulence in *C. parasitica* has not yet been successful in North America, even though it was shown that it might succeed in less diverse American populations (Scibilia & Shain, 1989). The failure can be ascribed to the high level of VCG diversity present in these populations (Anagnostakis & Kranz, 1987; Cortesi *et al.*, 1996; Milgroom *et al.*, 1991; Milgroom *et al.*, 1992), which results in ineffective spread of the hypovirus.

Control of *C. cubensis* using hypovirulence must still be tested in the field. The recent successful transfection of a *C. cubensis* isolate with a dsRNA hypovirus from *C. parasitica* (Chen *et al.*, 1996), indicates that hypovirulence remains a feasible control

measure, even though the fungus is represented by numerous VCGs in countries such as Venezuela, Brazil and Indonesia (Van Heerden, *et al.*, 1997; Van Zyl *et al.*, 1998), which would limit the utility of hypovirulence. In South Africa, the genetic diversity of *C. cubensis* is lower than it is in South America and Asia (Van Heerden, 1999; Wingfield *et al.*, 1997), but several VCGs still exist. The possibility of hypovirus transfer between vegetatively incompatible strains from this population (Van Heerden, 1999), could result in success with hypovirulence to control *C. cubensis* in South Africa.

POPULATIONS AND PHYLOGENY OF *CRYPHONECTRIA CUBENSIS*

Cryphonectria cubensis was reported for the first time from South Africa in 1989 (Wingfield *et al.*, 1989). The fungus causes cankers on *Eucalyptus* trees, which can girdle the stem and result in tree death. Since its first discovery, the fungus has been found in almost all areas in South Africa where *Eucalyptus* spp. and hybrids are commercially grown (Conradie *et al.*, 1990; Myburg, 1997; Van Zyl, 1995). It is consequently important to study *C. cubensis* in South Africa as well as in other countries, at the population level. Data obtained from these studies will be valuable in determining the area of origin of South African *C. cubensis*, as well as in the development of management strategies for Cryphonectria canker.

Cryphonectria cubensis occurs in diverse geographic areas, that mainly include tropical and sub-tropical regions of the world. The fungus is known in several South American countries, such as Venezuela (Van Heerden *et al.*, 1997), Brazil (Alfenas *et al.*, 1983; Hodges *et al.*, 1979; Van Zyl *et al.*, 1998) and Colombia (Chapter II, *this*

thesis). It also occurs in South East Asian regions, including Australia (Davison & Coates, 1991), Indonesia (Van Heerden *et al.*, 1997) and China (Hodges & Peterson, 1986).

In South Africa, *C. cubensis* causes basal cankers on eucalypts. It preferentially attacks young trees and causes death within the first year of growth (Wingfield *et al.*, 1989). Cankers in South Africa are usually covered with pycnidia, which represent the asexual fruiting structures. Sexual reproduction of the fungus is rare in South Africa (Wingfield *et al.*, 1997). In contrast, sexual reproduction is the common form of dispersal in other countries, where cankers are usually covered with both perithecia as well as pycnidia (Hodges *et al.*, 1979). Also, cankers occur much higher up on eucalypt stems, and can be found on much older trees than in South Africa. When these facts are considered, it appears that the fungus in South Africa is distinct from *C. cubensis* in South America and South East Asia.

Several authors have addressed the question whether South African *C. cubensis* is distinct from the fungus in other countries. These studies included determination of population diversity of the fungus in South Africa (Van Heerden, 1999), South America (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998) and Asia (Van Heerden *et al.*, 1997), as well as phylogenetic analyses using internal transcribed spacer (ITS) sequence data (Myburg, 1997; Myburg *et al.*, 1999). Data collected in this manner suggested that *C. cubensis* was introduced into South Africa, since the population with the lowest level of variation can be found in this country. Phylogenetic data imply that the origin of South African *C. cubensis* was in South America, since South

African isolates grouped together with South American isolates, but are more distinct from isolates originating in Australia, Indonesia and China.

In the past, only VCGs have been used for studying population diversity in *C. cubensis*, while only ITS sequence data were used for determining phylogenetic relationships. There is consequently an urgent need to develop molecular techniques for population studies of the fungus. Phylogenetic inferences may also be more useful when other, more variable regions of the genome are analysed. These are, for example, the intronic regions of the histone H3 gene (Steenkamp *et al.*, 1999), as well as the β-tubulin gene (Harrington & Rizzo, 1999; O'Donnell *et al.*, 1998; Thon & Royse, 1999). Future studies should also focus on elucidating the mating system of *C. cubensis*, in order to allow a more meaningful interpretation of population and phylogenetic data.

SUMMARY

- A vast array of molecular techniques are currently being used to study fungal population diversity. The most important of these include isozymes, RAPDs, RAMS, AFLPs, RFLPs and microsatellites. Of these techniques, only isozyme analysis has been used on *C. cubensis*.

- Vegetative incompatibility, a phenotypic character that can be tested *in vitro*, provides an easy and effective technique for the determination of fungal population diversity. This character is controlled by a small number of genes, and does not necessarily reflect the true variation present in a population. All of the published population studies on *C. cubensis* have used vegetative incompatibility tests. Data obtained from these studies have already been useful in elucidating the origin of *C. cubensis* in South Africa, and also in improving tree breeding strategies.

- The diversity of the South African *C. cubensis* population is lower than that of the fungus in other countries. This and other facts, suggest that the fungus is an introduced pathogen in South Africa.

- *Cryphonectria cubensis* in South Africa is very closely related to the fungus in South America, based on ITS DNA sequence data. It is also distinct from isolates originating in South East Asia. These facts suggest that South America is the possible origin of *C. cubensis* in South Africa.

REFERENCES

Adams, G., Johnson, N., Leslie, J. F. & Hart, L. P. (1987). Heterokaryons of *Gibberella zeae* formed following hyphal anastomosis or protoplast fusion. *Experimental Mycology* **11**, 339-353.

Alfenas, A. C., Jeng, R. & Hubbes, M. (1983). Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**, 197-205.

Alfenas, A. C., Jeng, R. & Hubbes, M. (1984). Isozyme and protein patterns of isolates of *Cryphonectria cubensis* differing in virulence. *Canadian Journal of Botany* **62**, 1756-1762.

Anagnostakis, S. L. (1977). Vegetative incompatibility in *Endothia parasitica*. *Experimental Mycology* **1**, 306-316.

Anagnostakis, S. L. (1982a). Biological control of chestnut blight. *Science* **215**, 466-472.

Anagnostakis, S. L. (1982b). Genetic analysis of *Endothia parasitica*: linkage data for four single genes and three vegetative compatibility types. *Genetics* **102**, 25-28.

Anagnostakis, S. L. (1984). Nuclear gene mutations in *Endothia* (*Cryphonectria*) *parasitica* that affect morphology and virulence. *Phytopathology* **74**, 561-565.

Anagnostakis, S. L., Hau, B. & Kranz, J. (1986). Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. *Plant Disease* **70**, 536-538.

Anagnostakis, S. L. & Kranz, J. (1987). Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. *Phytopathology* 77, 751-754.

Anderson, W. W., Berisford, C. W. & Kimmich, R. H. (1979). Genetic differences among five populations of the southern pine beetle. *Annals of the Entomological Society of America* **72**, 323-327.

Baleiras Couto, M. M., Hartog, B. J., Huis in't Veld, J. H. J., Hofstra, H. & Van der Vossen, J. M. B. M. (1996). Identification of spoilage yeasts in a food-production chain by microsatellite polymerase chain reaction fingerprinting. *Food Microbiology* **13**, 59-67.

Bayman, P. & Cotty, P. J. (1991). Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. *Canadian Journal of Botany* **69**, 1707-1711.

Beisson-Schecroun, J. (1962). Incompatibilité cellulaire et interactions nucléo-cytoplasmiques dans les phénomènes de barrage chez le *Podospora anserina*. *Annales de Genetique* **4**, 3-50.

Bernet, J. (1965). Mode d'action des gènes de barrage et relation entre l'incompatibilité cellulaire et l'incompatibilité sexuelle chez *Podospora anserina*. *Annales des Sciences Naturelles, Botanique, Paris* **12**, 611-768.

Bonfante, P., Lanfranco, L., Cometti, V. & Genre, A. (1997). Inter- and intraspecific variability in strains of the ectomycorrhizal fungus *Suillus* as revealed by molecular techniques. *Microbiological Research* **152**, 287-292.

Brasier, C. M. (1984). Inter-mycelial recognition systems in *Ceratocystis ulmi*: their physiological properties and ecological importance. In *The Ecology and Physiology of the Fungal Mycelium*, pp. 451-498. Edited by D. H. Jennings & A. D. M. Rayner. Cambridge: Cambridge University Press.

Burdon, J. J. & Roelfs, A. P. (1985a). The effect of sexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* **75**, 1068-1073.

Burdon, J. J. & Roelfs, A. P. (1985b). Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. *Phytopathology* **75**, 907-913.

Chan, K. F. & Sun, M. (1997). Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. *Theoretical and Applied Genetics* **95**, 865-873.

Chen, B., Chen, C., Bowman, B. H. & Nuss, D. L. (1996). Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogenic fungi phylogenetically related to *Cryphonectria parsitica*. *Phytopathology* **86**, 301-310.

Chen, R. S. & McDonald, B. A. (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics* **142**, 1119-1127.

Chen, R.-S., Boeger, J. M. & McDonald, B. A. (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**, 209-218.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990). Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**, 43-49.

Correll, J. C., Gordon, T. R. & McCain, A. H. (1992). Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Phytopathology* **82**, 415-420.

Cortesi, P., Milgroom, M. G. & Bisiach, M. (1996). Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. *Mycological Research* **100**, 1087-1093.

Davis, M. J. J., Bailey, C. S. & Smith, C. K. (1997). Increased informativeness of RAPD analysis by detection of microsatellite motifs. *BioTechniques* **23**, 285-290.

Davison, E. M. & Coates, D. J. (1991). Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157-160.

Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A. & Anagnostakis, S. L. (1977). Double-stranded RNA in *Endothia parasitica*. *Phytopathology* **67**, 1393-1396.

Delmotte, F., Bucheli, E. & Shykoff, J. A. (1999). Host and parasite population structure in a natural plant-pathogen system. *Heredity* **82**, 300-308.

Elliston, J. E. (1985). Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. *Phytopathology* **75**, 151-158.

Fahima, T., Kazmierczak, P., Hansen, D. R., Pfeiffer, P. & Van Alfen, N. K. (1993). Membrane-associated replication of an unencapsidated double-strand RNA of the fungus, *Cryphonectria parasitica*. *Virology* **195**, 81-89.

Department of Water Affairs and Forestry (1998). *Abstract of South African Forestry Facts of the Year 1996/1997.* South Africa: Forest Owners Association.

Francis, D. M. & St Clair, D. A. (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. *Current Genetics* **24**, 100-106.

Geistlinger, J., Weising, K., Kaiser, W. J. & Kahl, G. (1997). Allelic variation at a hypervariable compound microsatellite locus in the ascomycete *Ascochyta rabiei*. *Molecular and General Genetics* **256**, 298-305.

Glass, N. L. & Kuldau, G. A. (1992). Mating type and vegetative incompatibility in filamentous Ascomycetes. *Annual Review of Phytopathology* **30**, 201-224.

Goldstein, D. B., Linares, A. R., Cavalli-Sforza, L. L. & Feldman, M. W. (1995). An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139**, 463-471.

Goodwin, S. B., Drenth, A. & Fry, W. E. (1992). Cloning and genetic analysis of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Current Genetics* **22**, 107-115.

Grist, S. A., Firgaira, F. A. & Morley, A. A. (1993). Dinucleotide repeat polymorphism isolated by the polymerase chain reaction. *BioTechniques* **15**, 304-309.

Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995). A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied and Environmental Microbiology* **61**, 3943-9.

Hantula, M., Dusabenyagasani, M. & Hamelin, R. C. (1996). Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* **26**, 159-166.

Harrington, T. C. & Rizzo, D. M. (1999). Defining species in the fungi. In *Structure and dynamics of fungal populations*, pp. 43-71. Edited by J. J. Worrall: Kluwer Academic Press.

Hartl, D. L. & Clark, A. G. (1989). *Principles of population genetics*, Second Edition. Sunderland, Massachusetts, USA: Sinauer Associates, Inc.

Heiniger, U. & Rigling, D. (1994). Biological control of chestnut blight in Europe. *Annual Review of Phytopathology* **32**, 581-599.

Hill, M., Witsenboer, H., Zabeau, M., Vos, P., Kesseli, R. & Michelmore, R. (1996). PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics* **93**, 1202-1210.

Hillman, B. I., Shapira, R. & Nuss, D. L. (1990). Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. *Phytopathology* **80**, 950-956.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

Hodges, C. S. & Peterson, R. (1986). Forest pathology in the People's Republic of China. Observations of a forest pathology team.

Jarne, P. & Lagoda, P. J. L. (1996). Microsatellites, from molecules to populations and back. *TREE* **11**, 424-429.

Jeffreys, A. J., Wilson, V. & Thein, S. L. (1985). Individual-specific 'fingerprints' of human DNA. *Nature* **316**, 76-79.

Lagerkrantz, U., Ellegren, H. & Andersson, L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**, 1111-1115.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology* **31**, 127-150.

Liu, Y. & Milgroom, M. G. (1996). Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* **86**, 79-86.

McDonald, B. A. & McDermott, J. M. (1993). Population genetics of plant pathogenic fungi. *BioScience* **43**, 311-319.

McDonald, B. A., Miles, J., Nelson, L. R. & Pettway, R. E. (1994). Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology* **84**, 250-255.

Meyer, W., Marszewska, K., Amirmostofian, M., Igreja, R. P., Hardtke, C., Methling, K., Viviani, M. A., Chindamporn, A., Sukroongreung, S., John, M. A., Ellis, D. H. & Sorrell, T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis* **20**, 1790-1799.

Milgroom, M. G. (1995). Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany* **73(Suppl. 1)**, S311-S319.

Milgroom, M. G. & Lipari, S. E. (1995). Population differentiation in the chestnut blight fungus, *Cryphonectria parasitica*, in Eastern North America. *Phytopathology* **85**, 155-160.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y. (1993). Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **70**, 385-392.

Milgroom, M. G., Lipari, S. E. & Wang, K. (1992). Comparison of genetic diversity in the chestnut blight fungus, *Cryphonectria* (*Endothia*) *parasitica*, from China and the U.S. *Mycological Research* **96**, 1114-1120.

Milgroom, M. G., MacDonald, W. L. & Double, M. L. (1991). Spatial pattern analysis of vegetative compatibility groups in the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany* **69**, 1407-1413.

Moon, C. D., Tapper, B. A. & Scott, B. (1999). Identification of *Epichloë* endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**, 1268-1279.

Myburg, H. (1997). *Cryphonectria cubensis*, a molecular taxonomic and population study. Department of Microbiology and Biochemistry. Bloemfontein: University of the Orange Free State. *M.Sc. Thesis*.

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

Newhouse, J. R. & MacDonald, W. L. (1991). The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Canadian Journal of Botany* **69**, 602-614.

Newhouse, J. R., MacDonald, W. L. & Hoch, H. C. (1990). Virus-like particles in hyphae and conidia of European hypovirulent (dsRNA-containing) strains of *Cryphonectria parasitica*. *Canadian Journal of Botany* **68**, 90-101.

O'Donnell, K., Cigelnik, E. & Nirenberg, H. I. (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**, 465-493.

Panaud, O., Chen, X. & McCough, R. (1995). Frequency of microsatellite sequences in rice (*Oryza sativa* L.). *Genome* **38**, 1170-1176.

Perkins, D. D., Radford, A., Newmeyer, D. & Bjorkman, M. (1982). Chromosomal loci of *Neurospora crassa*. *Microbiological Reviews* **46**, 426-570.

Puhalla, J. E. & Mayfield, J. E. (1974). The mechanism of heterokaryotic growth in *Verticillium dahliae*. *Genetics* **76**, 411-422.

Puterka, G. J., Black, W. C., Steiner, W. M. & Burton, R. L. (1993). Genetic variation and phylogenetic relationships among worldwide collections of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), inferred from allozyme and RAPD-PCR markers. *Heredity* **70**, 604-618.

Rae, B. P., Hillman, B. I., Tartaglia, J. & Nuss, D. L. (1989). Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. *The EMBO Journal* **8**, 657-663.

Rafalski, J. A., Vogel, J. M., Morgante, M., Powell, W., Andre, C. & Tingey, S. V. (1996). Generating and using DNA markers in plants. In *Analysis of non-mammalian genomes - a practical guide*, pp. 75-134. Edited by B. Birren & E. Lai. New York: Academic Press.

Rigling, D. & Van Alfen, N. K. (1991). Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. *Journal of Bacteriology* **173**, 8000-8003.

Scibilia, K. L. & Shain, L. (1989). Protection of American chestnut with hypovirulent conidia of *Cryphonectria* (*Endothia*) *parasitica*. *Plant Disease* **73**, 840-843.

Slatkin, M. (1995). A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457-462.

Steenkamp, E. T., Wingfield, B. D., Coutinho, T. A., Wingfield, M. J. & Marasas, W. F. O. (1999). Differentiation of *Fusarium subglutinans* f.sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**, 3401-3406.

Struss, D. & Plieske, J. (1998). The use of microsatellite markers for detection of genetic diversity in barley populations. *Theoretical and Applied Genetics* **97**, 308-315.

Thon, M. R. & Royse, D. J. (1999). Partial β-tubulin gene sequences for evolutionary studies in the Basidiomycotina. *Mycologia* **91**, 468-474.

Turgeon, B. G., Christiansen, S. K. & Yoder, O. C. (1993). Mating type genes in ascomycetes and their imperfect relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*, pp. 199-215. Edited by D. R. Reynolds & J. W. Taylor. Wallingford: CAB International.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis.*

Van Heerden, S. W., Wingfield, M. J., Coutinho, T. & Van Zyl, L. M. (1997). Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv. (Abstract).

Van Zyl, L. M. (1995). Some factors affecting the susceptibility of *Eucalyptus* spp. to *Cryphonectria cubensis*. Department of Microbiology and Biochemistry. Bloemfontein: University of the Orange Free State. *M.Sc. Thesis.*

Van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998). Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**, 41-47.

Whisson, S. C., Drenth, A., Maclean, D. J. & Irwin, J. A. (1994). Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Current Genetics* **27**, 77-82.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

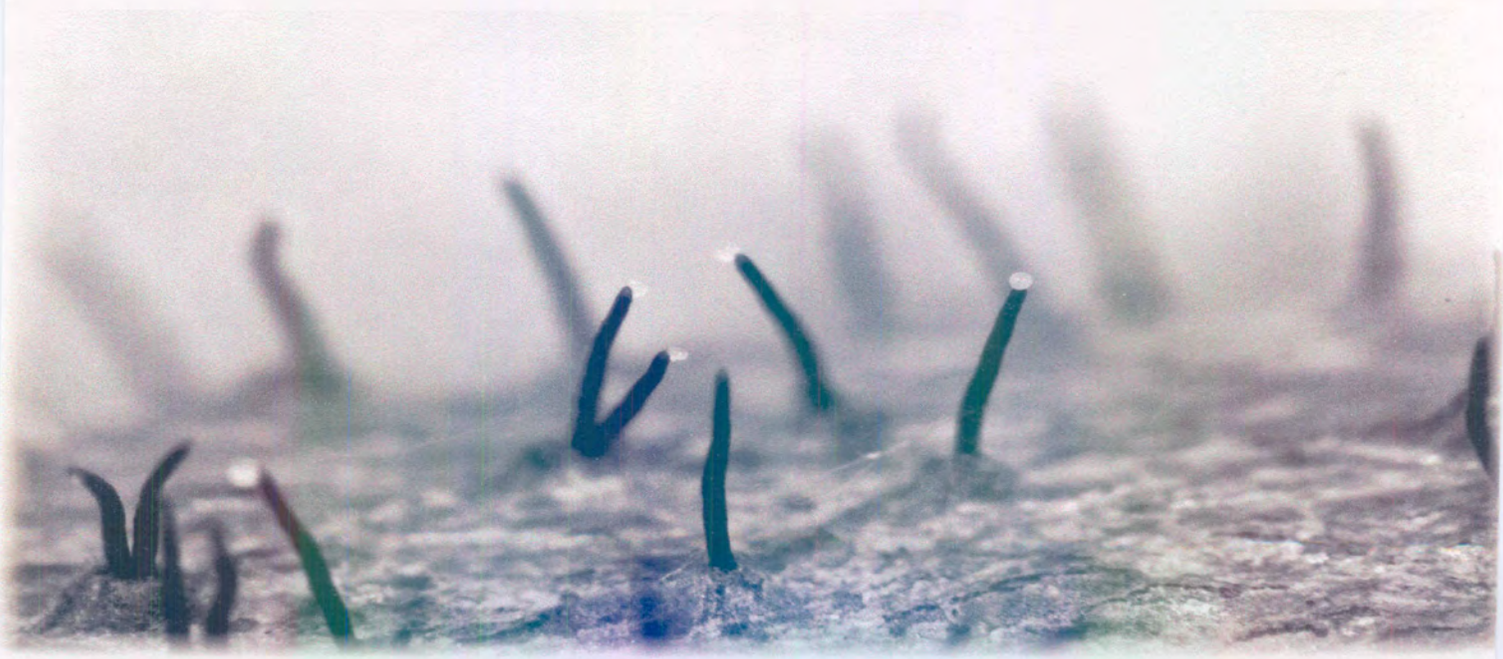
Wingfield, M. J. (1990). Current status and future prospects of forest pathology in South Africa. *South African Journal of Science* **86**, 60-62.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Wingfield, M. J., Van Zyl, L. M., Van Heerden, S., Myburg, H. & Wingfield, B. D. (1997). Virulence and the genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In *Physiology and genetics of tree-phytophage interactions*, pp. 163-172. Edited by F. Lieutier, W. J. Mattson & M. R. Wagner. Gujan, France: INRA Editions.

CHAPTER II

PHYLOGENETIC RELATIONSHIPS OF *CRYPHONECTRIA CUBENSIS* FROM COLOMBIA



PHYLOGENETIC RELATIONSHIPS OF *CRYPHONECTRIA CUBENSIS* FROM COLOMBIA

ABSTRACT

Cryphonectria cubensis is a pathogen of *Eucalyptus* that causes girdling stem cankers. Identification of this fungus is complicated by a number of inconsistencies in the morphology and biology of isolates from different parts of the world. Perithecia representing the teleomorph are common in South American populations, but these structures have only been observed once in South Africa, where pycnidia are predominant on cankers. Recent studies based on ITS sequence data, have shown that *C. cubensis* from Venezuela, Brazil, Australasia and South Africa are phylogenetically closely related, and that they form a monophyletic group. Following the recent acquisition of a putative *C. cubensis* population from Colombia, we have initiated studies to determine the phylogenetic relationships of these isolates in relation to previously studied isolates from geographically diverse origins. The internal transcribed spacer (ITS) region of the ribosomal DNA operon was PCR amplified with two specific primers. The amplification products were sequenced and the sequence data subjected to phylogenetic analyses using parsimony. The Colombian *C. cubensis* isolates grouped together with isolates from South Africa, Brazil and Venezuela, and differs from isolates originating in South East Asia. This finding confirms that the fungus is typical of *C. cubensis*, and that Colombian isolates reside in the South American clade.

INTRODUCTION

Cryphonectria cubensis is the causal agent of Cryphonectria canker that occurs on the stems of *Eucalyptus* trees (Florence *et al.*, 1986; Hodges *et al.*, 1979; Wingfield *et al.*, 1989). This disease is a significant threat to commercial *Eucalyptus* plantations in tropical and sub-tropical countries, since severe infection can lead to tree death (Conradie *et al.*, 1992; Swart *et al.*, 1992; Wingfield *et al.*, 1989). *C. cubensis* also occurs on trees other than *Eucalyptus* (Hodges *et al.*, 1986; Myburg *et al.*, 1999; Swart *et al.*, 1991), and some of these might reflect original native hosts. For example, its occurrence on clove (*Eugenia carophyllus*) (Alfenas *et al.*, 1984; Hodges *et al.*, 1986; Micales & Stipes, 1984; Micales *et al.*, 1987) has lead to a suggestion that *C. cubensis* could have originated in Indonesia, where clove is native.

In South Africa, *C. cubensis* causes basal cankers on young *Eucalyptus* trees during the first few years of growth (Wingfield *et al.*, 1989). Conversely, cankers caused by the fungus typically occur higher up on stems of mature *Eucalyptus* trees in South America and South East Asia (Florence *et al.*, 1986; Hodges *et al.*, 1979; Sharma *et al.*, 1985). Cankers in the latter areas are consistently covered with perithecia, which suggests that sexual reproduction is the predominant mode of reproduction. Cankers in South Africa very rarely display perithecia, but are covered with pycnidia (Van Heerden, 1999; Wingfield *et al.*, 1997), suggesting that asexual reproduction is predominant. These dissimilarities between the South African and other forms of *C. cubensis* has prompted an investigation into the relatedness of isolates of the fungus from diverse geographic origins.

The phylogenetic relationships between isolates of *C. cubensis* originating in South Africa, South America, Asia and Australia have recently been elucidated (Myburg *et al.*, 1999). This study involved phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) operon. Results indicated that all of the investigated isolates of *C. cubensis* were very closely related. However, two separate clades could be identified. Isolates from South America and South Africa represented a clade separate from those originating in South East Asia. It was thus hypothesised that *C. cubensis* has probably been introduced into South Africa from South America.

Even though both molecular (Davison & Coates, 1991; Myburg, 1997; Myburg *et al.*, 1999) and phenotypic data (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998) are available for many isolates of *C. cubensis* from South Africa and South America, several questions remain unresolved. *C. cubensis* populations in South American countries, for example Brazil and Venezuela, display very high levels of diversity (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998). This information makes it difficult to identify a potential area of origin of the South African *C. cubensis* population. Recent molecular data on the diversity of the South African *C. cubensis* population suggests that genotypic variation has been underestimated in the past (Chapter VI, *this thesis*), where vegetative incompatibility was used to measure variation. It has also been suggested that the area of origin of the South African *C. cubensis* population might not be in South America as previously speculated (Chapter V, *this thesis*). These facts indicate that molecular data sets pertaining to the relatedness and diversity of *C. cubensis* from various parts of the world should be increased, in order to improve the current definition of this species.

The aim of this study was to verify the identity of putative *C. cubensis* isolates recently collected in Colombia. Phylogenetic analyses using parsimony were performed on the ITS DNA sequence of several Colombian *C. cubensis* isolates, and compared to previously published ITS sequences of *C. cubensis* from South America, South Africa, and South East Asia. Sequence data from the apparently closely related species, *Cryphonectria parasitica*, were included to test its relatedness to Colombian *C. cubensis*.

MATERIALS AND METHODS

Fungal isolates and DNA isolation

Genomic DNA was isolated from four isolates of *C. cubensis* from Colombia, namely CRY1209, CRY1242, CRY1318, and CRY1394, using the protocol described by Raeder & Broda (1985). Additionally, ITS sequence data for the isolates used by Myburg *et al.* (1999) were obtained from Genbank (Table 1). These previously studied isolates include three of *Cryphonectria parasitica* and one *Diaporthe ambigua*. All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

PCR amplification

The ITS1, 5·8S and ITS2 regions of the rDNA operon were amplified using primers ITS1 and ITS4 (White *et al.*, 1990) with 25 ng of genomic DNA from each of the four Colombian *C. cubensis* isolates. Each PCR reaction also included 5 µl 10x PCR

Reaction Buffer, 4·5 mM $MgCl_2$, 250 µM of each dNTP, 5U Expand® *Taq* DNA Polymerase (Boehringer Mannheim, GmbH) and 0·5 µM of each primer. Reaction mixtures were adjusted to 25 µl with the addition of sterile water, and overlaid with mineral oil to prevent evaporation during thermal cycling. The PCR protocol involved an initial denaturation at 95°C for 1 min, followed by 35 cycles of primer annealing at 55°C (45 s), DNA elongation at 72°C (2 min) and denaturation at 95°C (30 s). The reaction was completed with a final extension at 72°C for 7 min. Ten µl of each PCR amplification product was electrophoresed on a 1% w/v agarose gel (6 V/cm for 1 h 30 min) to confirm the presence of a single band at *ca.* 500 bp (base pairs).

Sequencing and data analyses

PCR amplification products were purified using a Nucleospin Extract PCR Purification Kit (Macherey Nagel, GmbH) and sequenced using a Dye Terminator Cycle Sequencing Kit (Perkin Elmer, CA) and automated sequencer. Electropherograms were analysed using the Sequence Navigator computer software, followed by manual alignment with previously published sequences from *C. parasitica* and *D. ambigua* (Myburg, *et al.*, 1999). Aligned sequences were subjected to phylogenetic analyses using parsimony (PAUP* 4.0) (Swofford, 1998). The sequence from *D. ambigua* was used as an outgroup with a heuristic search using TBR (tree bisection reconnection) of the data set. Bootstrap analysis (1000 replicates) was used to determine the confidence of each branch.

RESULTS

PCR amplification

Amplification of the ITS rDNA region using PCR, yielded a *ca.* 500 bp (base pair) product for all four Colombian *C. cubensis* isolates. This region included the variable ITS1 and ITS2 spacer regions, as well as the conserved 5·8S gene.

Sequencing and data analyses

Automated sequencing of purified ITS PCR amplification products resulted in a readable sequence of *ca.* 500 bp for all four Colombian *C. cubensis* isolates. Manual alignment of ITS sequence data resulted in a total of 577 characters (Figure 1).

Heuristic analysis of the 577 aligned characters generated one most parsimonious tree (Figure 2). Three clades were identified from the phylogenetic tree. One clade was comprised of three *C. parasitica* isolates, which grouped more closely to the *C. cubensis* isolates than to the outgroup taxon, *D. ambigua*. The *C. cubensis* isolates grouped into two separate clades that reflect their geographic origin. One clade included *C. cubensis* isolates from South America and South Africa, while the other clade was represented by isolates from South East Asia. The four Colombian isolates of *C. cubensis* grouped within the South American clade.

Three signature sequences, within close proximity of each other, are present in the ITS2 region of the rDNA operon (Figure 1). These sequences occur at characters 452,

459-460, and 486. In all cases, *C. cubensis* isolates from South America, including Colombia, and South Africa differed from South East Asian isolates by the substitution of a single base. At character 452, a guanine in South East Asian isolates was substituted for an adenine in South American isolates. Similarly, at characters 459-460, a tyrosine was substituted for a cytosine, while character 486 displays the same substitution as 452.

DISCUSSION

In this study, it was confirmed that recently collected isolates from *Eucalyptus* cankers in Colombia are typical of *C. cubensis*. The isolates are also phylogenetically closely related to other isolates of the fungus from South America. This finding suggests that the South American *C. cubensis* isolates had a common ancestor, which is consistent with the findings of Myburg *et al.* (1999).

Phylogenetic analysis supported the grouping of *C. cubensis* isolates into two distinct clades, namely a South American and a South East Asian clade (Myburg *et al.*, 1999). Therefore, based on ITS DNA sequence, isolates that represent the two clades are distinct. An isolate from South Africa grouped within the South American clade, suggesting that *C. cubensis* from South Africa and South America are closely related. However, recent evidence obtained using polymorphic markers, suggest that this is not the case, since South African *C. cubensis* isolates can be separated from those originating in South America (Chapter 5, *this thesis*). Consequently, it appears that the ITS rDNA data are not sufficiently variable to distinguish between South African and South American isolates of *C. cubensis*. The resolution of molecular data can be

improved by using more variable regions of the genome, for example the intron sequences of the histone H3 gene (Steenkamp *et al.*, 1999) and the β-tubulin gene (O'Donnell *et al.*, 1998; Thon & Royse, 1999).

Three signature sequences were observed in the ITS2 region of the isolates used in this study. All three sequences clearly differentiate *C. cubensis* isolates from South America and South East Asia. These sequences can thus be used as robust signatures of isolates from these regions of the world, and would be useful in the development of diagnostic techniques to distinguish between *C. cubensis* isolates from different countries. Myburg *et al.* (1999) developed a restriction enzyme-based diagnostic procedure to distinguish between isolates of *C. cubensis*, *Endothia eugeniae*, *C. parasitica*, *Endothia gyrosa*, and *D. ambigua*. The signature sequences found in this study, however, will enable intra-specific differentiation of *C. cubensis* from diverse geographic origins.

Using ITS sequence data, we were able to show that Colombian isolates of *C. cubensis* are very similar to other isolates of this fungus from South American countries. This finding suggests that *C. cubensis* in Colombia might display the same properties as the fungus in Venezuela (Van Heerden *et al.*, 1997) and Brazil (Van Zyl *et al.*, 1998), at the population level. Future research on *C. cubensis* from Colombia should, therefore, include detailed population studies and an investigation into the mating system of the fungus prevalent in Colombia.

REFERENCES

Alfenas, A. C., Jeng, R. & Hubbes, M. (1984). Isozyme and protein patterns of isolates of *Cryphonectria cubensis* differing in virulence. *Canadian Journal of Botany* **62**, 1756-1762.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1992). Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. *European Journal of Forest Pathology* **22**, 312-315.

Davison, E. M. & Coates, D. J. (1991). Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157-160.

Florence, E. J. M., Sharma, J. K. & Mohanan, C. (1986). A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384-386.

Hodges, C. S., Alfenas, A. C. & Ferreira, F. A. (1986). The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**, 343-350.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

Micales, J. A. & Stipes, R. J. (1984). Differentiation of *Endothia* and *Cryphonectria* species by polyacrylamide gel electrophoresis. *Phytopathology* **74**, 883-884. (Abstract).

Micales, J. A., Stipes, R. J. & Bonde, M. R. (1987). On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. *Mycologia* **79**, 707-720.

Myburg, H. (1997). *Cryphonectria cubensis*, a molecular taxonomic and population study. Department of Microbiology and Biochemistry. Bloemfontein: University of the Orange Free State. *M.Sc. Thesis.*

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

O'Donnell, K., Cigelnik, E. & Nirenberg, H. I. (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**, 465-493.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**, 17-20.

Sharma, J. K., Mohanan, C. & Florence, E. J. M. (1985). Occurrence of Cryphonectria canker disease of *Eucalyptus* in Kerala, India. *Annals of Applied Biology* **106**, 265-276.

Steenkamp, E. T., Wingfield, B. D., Coutinho, T. A., Wingfield, M. J. & Marasas, W. F. O. (1999). Differentiation of *Fusarium subglutinans* f.sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**, 3401-3406.

Swart, W. J., Conradie, E. & Wingfield, M. J. (1991). *Cryphonectria cubensis*, a potential pathogen of *Psidium guajava* in South Africa. *European Journal of Forest Pathology* **21**, 424-429.

Swart, W. J., Conradie, E., Wingfield, M. J. & Venter, W. B. (1992). Effects of water stress on the development of cambial lesions caused by *Cryphonectria cubensis* on *Eucalyptus grandis*. *Plant Disease* **76**, 744-746.

Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Sunderland, Massachusetts: Sinauer Associates.

Thon, M. R. & Royse, D. J. (1999). Partial β-tubulin gene sequences for evolutionary studies in the Basidiomycotina. *Mycologia* **91**, 468-474.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis*.

Van Heerden, S. W., Wingfield, M. J., Coutinho, T. & Van Zyl, L. M. (1997). Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv. (Abstract).

Van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998). Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**, 41-47.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications.*, pp. 315-322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Wingfield, M. J., Van Zyl, L. M., Van Heerden, S., Myburg, H. & Wingfield, B. D. (1997). Virulence and the genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In *Physiology and genetics of tree-phytophage interactions.*, pp. 163-172. Edited by F. Lieutier, W. J. Mattson & M. R. Wagner. Gujan, France: INRA Editions.

Table 1. *Cryphonectria cubensis* isolates used in this study.

Isolate Identity	Isolate number[a]	Country of Origin[b]	Genbank Accession Number
Cryphonectria cubensis	CRY 1209	Colombia	AF172654
..	CRY 1242	..	AF172655
..	CRY 1318	..	AF172656
..	CRY 1394	..	AF172657
..	CRY 0129	Brazil	AF046900
..	CRY 0138	..	AF046891
..	CRY 0268	Venezuela	AF046897
..	CRY 0140	South Africa	AF046892
..	CRY 0289	Indonesia	AF046896
..	CRY 0127	China	AF046890
..	CRY 0082	Thailand	AF046899
..	CRY 0046	Australia	AF046893
..	CRY 0033	..	AF046894
..	CRY 0126	..	AF046895
Cryphonectria parasitica	CRY 0067	USA	AF046903
..	CRY 0044	USA	AF046902
..	CRY 0066	USA	AF046901
Diaporthe ambigua	CMW 2498	Netherlands	AF046906

[a] All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

[b] Isolates from Colombia were sequenced in this study. Data pertaining to other isolates originated from the study of Myburg *et al.* (1999).

Figure 1. Raw sequence data of the ITS1, 5·8S and ITS2 regions of the rDNA operon of *C. cubensis* isolates used in this study. Unknown characters are indicated with a question mark, while gaps in the sequence data are indicated with "-". Signature sequences are boxed.

```
[                  10         20         30         40         50         60         70]
[                   .          .          .          .          .          .          . ]

AF046900Br    CCCAGA--TA CCC-TTTGTG AA-CTTATA- CC-TTTT-TA T--------C GTTGCCTCGG CGCCGAGCTG    [55]
AF046896In    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046893Au    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046890Ch    ....A.--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046894Au    ....A.--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046895Au    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046899Th    ......--.. ...-...... ..-......- ..-....-.. .--------. ...CG..... .........C.   [55]
AF046891Br    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046897Ve    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046892SA    ?.....--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
3B3.7__CRY    ?????????? ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [57]
3B21.3_CRY    ?????????? ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [57]
3B19.3_CRY    ?????????? ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [57]
3B4.3__CRY    ......--.. ...-...... ..-......- ..-....-.. .--------. .......... .........C.   [55]
AF046903Cp    ?????????? ??.C--.... ..-......A ..A...--.. .--------. .......... ...T....CT   [57]
AF046902Cp    ?????????? ?????????? ????.....A ..A...--.. .--------. .......... ...T....CT   [60]
AF046901Cp    ?????????? ?????????? ?????....A ..A...--.. .--------. .......... ...T....CT   [60]
2498_D.amb    .....-CGC. ...----.A. ..A.CCT.TG TGGAAGCT.. .ACCTTA-CT .......... ...T-..-CT   [62]

[                  80         90         100        110        120        130        140]
[                   .          .          .          .          .          .          . ]

AF046900Br    GGAG------ ---------- ----TGCTCT TCTGTGC--- -------TCC C--------- ---------C   [77]
AF046896In    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046893Au    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046890Ch    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046894Au    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046895Au    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046899Th    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046891Br    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046897Ve    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046892SA    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
3B3.7__CRY    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [79]
3B21.3_CRY    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [79]
3B19.3_CRY    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [79]
3B4.3__CRY    ....------ ---------- ----...... .......--- -------... .--------- ---------.   [77]
AF046903Cp    CT------GG GGGGGGGTTG GCGAA.GCAG A--T.TTCTT CCTT--C... .CTCCCTCCC CCCCCTCTT.   [117]
AF046902Cp    CT------GG GGGGGGGTTG GCGAA.GCAG A--T.TTCTT CCTT--C... .CTCCCTCCC CCCCCTCTT.   [120]
AF046901Cp    CT------GG GGGGGGGTTG GCGAA.GCAG A--T.TTCTT CCTT--C... .CTCCCTCCC CCCCCTCTT.   [120]
2498_D.amb    ..TCCCTCGG GG-------- ---------- ---------- ---------- ---------- -CCCCT----   [79]
```

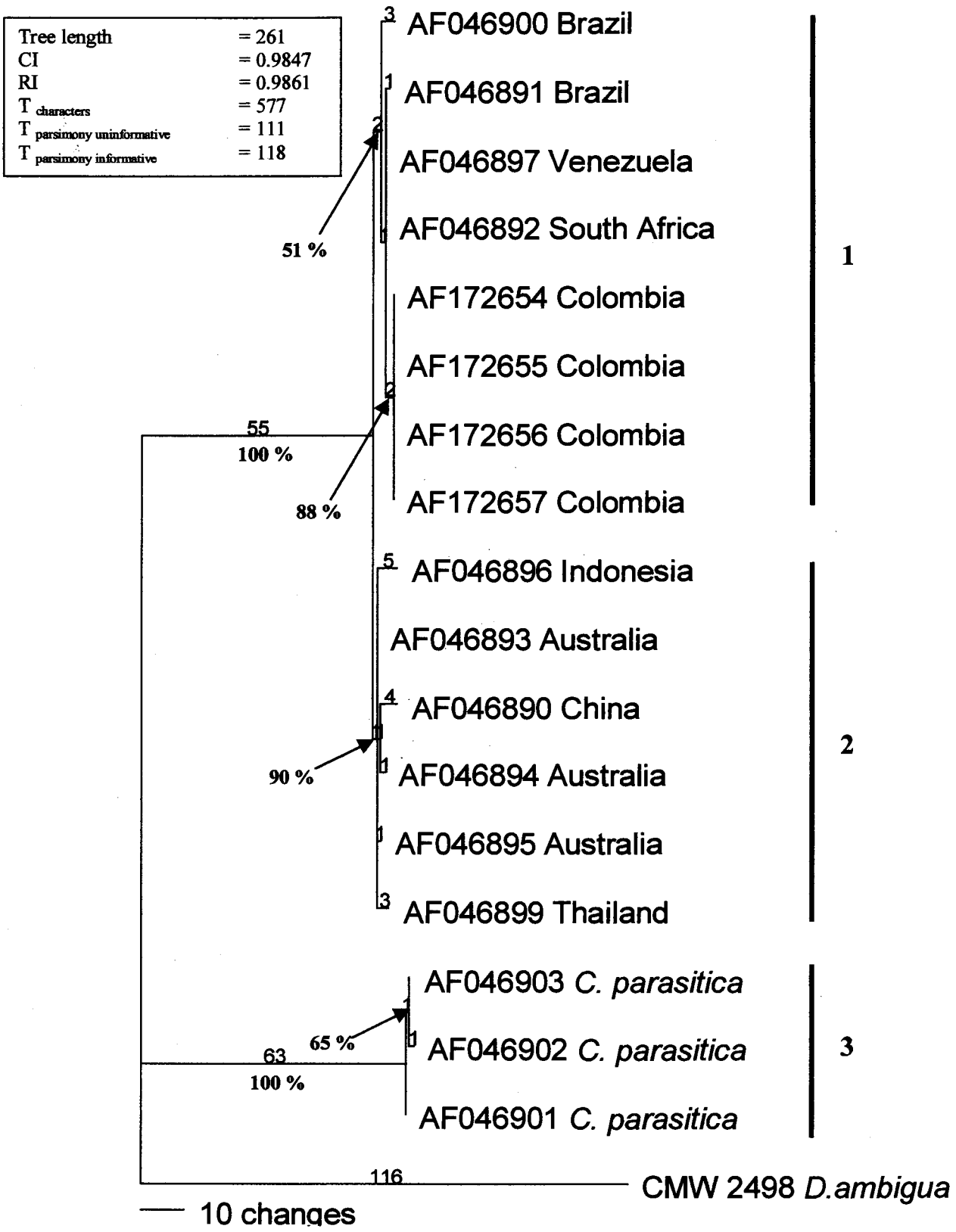
```
[                   150         160         170         180         190         200         210]
[                   .           .           .           .           .           .           .  ]

AF046900Br     CACC----GC GCAAGCAGT- -------GGA GCAGGCCCG- CCGGCGGCCC ACCAAA-CTC TTT-GTTTTT    [132]
AF046896In     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046893Au     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046890Ch     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046894Au     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046895Au     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046899Th     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046891Br     ....----.. .........- -------... .........- .......... ..T...-... ...-......    [132]
AF046897Ve     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046892SA     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
3B3.7__CRY     ....----.. .........- -------... .........- .......... ......-... ...-......    [134]
3B21.3_CRY     ....----.. .........- -------... .........- .......... ......-... ...-......    [134]
3B19.3_CRY     ....----.. .........- -------... .........- .......... ......-... ...-......    [134]
3B4.3__CRY     ....----.. .........- -------... .........- .......... ......-... ...-......    [132]
AF046903Cp     ....-----.T ....A.G..- TGTTGG-... .........- .......... ..T...-... ...-......    [178]
AF046902Cp     ....-----.T ....A.G..- TGTTGG-... .........- .......... ..T...-... ...-......    [181]
AF046901Cp     ....-----.T ....A.G..- TGTTGG-... .........- .......... ..T...-... ...-......    [181]
2498_D.amb     ....CT---- -----.G.G- TGTT----.. .ACA.....T .-.......A ...T..-... ..--......    [131]

[                   220         230         240         250         260         270         280]
[                   .           .           .           .           .           .           .  ]

AF046900Br     AGAA--CGT- ATCTCTTCTG AGTGTTTATA ACAAACAAA- TGAATCAAAA CTTTCAACAA CGGATCTCTT    [198]
AF046896In     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046893Au     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046890Ch     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046894Au     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046895Au     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046899Th     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046891Br     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046897Ve     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046892SA     ....--...- .......... .......... .........- .......... .......... ..........    [198]
3B3.7__CRY     ....--...- .......... .......... .........- .......... .......... ..........    [200]
3B21.3_CRY     ....--...- .......... .......... .........- .......... .......... ..........    [200]
3B19.3_CRY     ....--...- .......... .......... .........- .......... .......... ..........    [200]
3B4.3__CRY     ....--...- .......... .......... .........- .......... .......... ..........    [198]
AF046903Cp     .T..--.C.- .......... ...ACA..A. CA...A...A .......... .......... ..........    [245]
AF046902Cp     .T..--.C.- .......... ...ACA..A. CA...A...A .......... .......... ..........    [248]
AF046901Cp     .T..--.C.- .......... ...ACA..A. CA...A...A .......... .......... ..........    [248]
2498_D.amb     .??----C.G .AA--C.... ..CACAA-A. CAT..A---- .......... .......... ..........    [190]
```

```
[                290        300        310        320        330        340        350]
[                .          .          .          .          .          .          .  ]

AF046900Br    GGTTCTGGCA TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT   [268]
AF046896In    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046893Au    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046890Ch    .......... .......... .....C.C.. ....C....C .......... .......... ..........   [268]
AF046894Au    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046895Au    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046899Th    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046891Br    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046897Ve    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046892SA    .......... .......... .......... .......... .......... .......... ..........   [268]
3B3.7__CRY    .......... .......... .......... .......... .......... .......... ..........   [270]
3B21.3_CRY    .......... .......... .......... .......... .......... .......... ..........   [270]
3B19.3_CRY    .......... .......... .......... .......... .......... .......... ..........   [270]
3B4.3__CRY    .......... .......... .......... .......... .......... .......... ..........   [268]
AF046903Cp    .......... .......... .......... .......... .......... .......... .........G   [315]
AF046902Cp    .......... .......... .......... .......... .......... .......... .........G   [318]
AF046901Cp    .......... .......... .......... .......... .......... .......... .........G   [318]
2498_D.amb    .......... .......... .......... .......... .......... .......... .........G   [260]

[                360        370        380        390        400        410        420]
[                .          .          .          .          .          .          .  ]

AF046900Br    CGAATCTTTG AACGCACATT GCGCCCGTTG GAATTCCACC GGGCAT-GCC T-GTTCGAG- CGTCATTTCA   [335]
AF046896In    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046893Au    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046890Ch    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046894Au    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046895Au    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046899Th    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046891Br    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046897Ve    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
AF046892SA    .......... .......... .......... .......... ......-... .-.......- ..........   [335]
3B3.7__CRY    .......... .......... .......C.. ........G. ......-... .-.......- ..........   [337]
3B21.3_CRY    .......... .......... .......C.. ........G. ......-... .-.......- ..........   [337]
3B19.3_CRY    .......... .......... .......C.. ........G. ......-... .-.......- ..........   [337]
3B4.3__CRY    .......... .......... .......C.. ........G. ......-... .-.......- ..........   [335]
AF046903Cp    .......... .......... .---....-- .........- -.....-..? .-.......- ..........   [375]
AF046902Cp    .......... .......... .---....-- .........- -.....-... .-.......- ..........   [378]
AF046901Cp    .......... .......... .---....-- .........- -.....-... .-.......- ..........   [378]
2498_D.amb    .......... .......... .---....-- .........- -.....C... .-.......- ..........   [321]
```

```
[                  430         440         450         460         470         480         490]
[                  .           .           .           .           .           .           .  ]

AF046900Br    ACCCTCAAGC CTGGCTTGGT -TTGGGGCAC TACCTGTTT- ACAGCGGGTA GGCCCTGAAA -TTTAATGGC    [402]
AF046896In    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046893Au    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046890Ch    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046894Au    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046895Au    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046899Th    .......... .......... G......... .G.....T .......... .......... -....G....    [404]
AF046891Br    .......... .......... G......... ........C- .......... .......... -.........    [403]
AF046897Ve    .......... .......... G......... ........C- .......... .......... -.........    [403]
AF046892SA    .......... .......... G......... ........C- .......... .......... -.........    [403]
3B3.7__CRY    .......... .......... G......... ........C- .......... .......... -.........    [405]
3B21.3_CRY    .......... .......... G......... ........C- .......... .......... -.........    [405]
3B19.3_CRY    .......... .......... G......... ........C- .......... .......... -.........    [405]
3B4.3__CRY    .......... .......... G......... ........C- .......... .......... -.........    [403]
AF046903Cp    .......... T......... G......T.. ....C..AAA .---...-.. .......... -....G....    [440]
AF046902Cp    .......... T......... G......T.. ....C..AAA .---...-.. .......... C....G....    [444]
AF046901Cp    .......... T......... G......T.. ....C..AAA .---...-.. .......... -....G....    [443]
2498_D.amb    .......... .......... G......... .G.T.C..AC C..AGA?.C. .......... -....G....    [390]

[                  500         510         520         530         540         550         560]
[                  .           .           .           .           .           .           .  ]

AF046900Br    GGGCTCGCTA AGA-CTCTGA GCGTAGTAGT TTTTAT---- -CA-CCTCGC TTTGGAAGGA TTA-GCGG-T    [463]
AF046896In    .......... ...-...... A......... ......---- -..-...... .......... ...-CGA.-.    [465]
AF046893Au    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [465]
AF046890Ch    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [465]
AF046894Au    .......... ...-...... .......... ......---- -..-...... .......... ...--...-.    [464]
AF046895Au    .......... ...-...... .......... ......---- -..-...... .......... ...A....-.    [466]
AF046899Th    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [465]
AF046891Br    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [464]
AF046897Ve    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [464]
AF046892SA    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [464]
3B3.7__CRY    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [466]
3B21.3_CRY    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [466]
3B19.3_CRY    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [466]
3B4.3__CRY    .......... ...-...... .......... ......---- -..-...... .......... ...-....-.    [464]
AF046903Cp    .......... ...-...... ..C....... ....T.TTCT T..A...... .......... ...-....T.    [508]
AF046902Cp    .......... ...-...... ..C....... ....T.TTCT T..A...... .......... ...-....T.    [512]
AF046901Cp    .......... ...-...... .......... ....T.TTCT T..A...... .......... ...-....T.    [511]
2498_D.amb    .A.....-. G..-.C.C.. ...C...... .---------- AA.C...... .C.......C CCT-.GC.G.    [448]
```

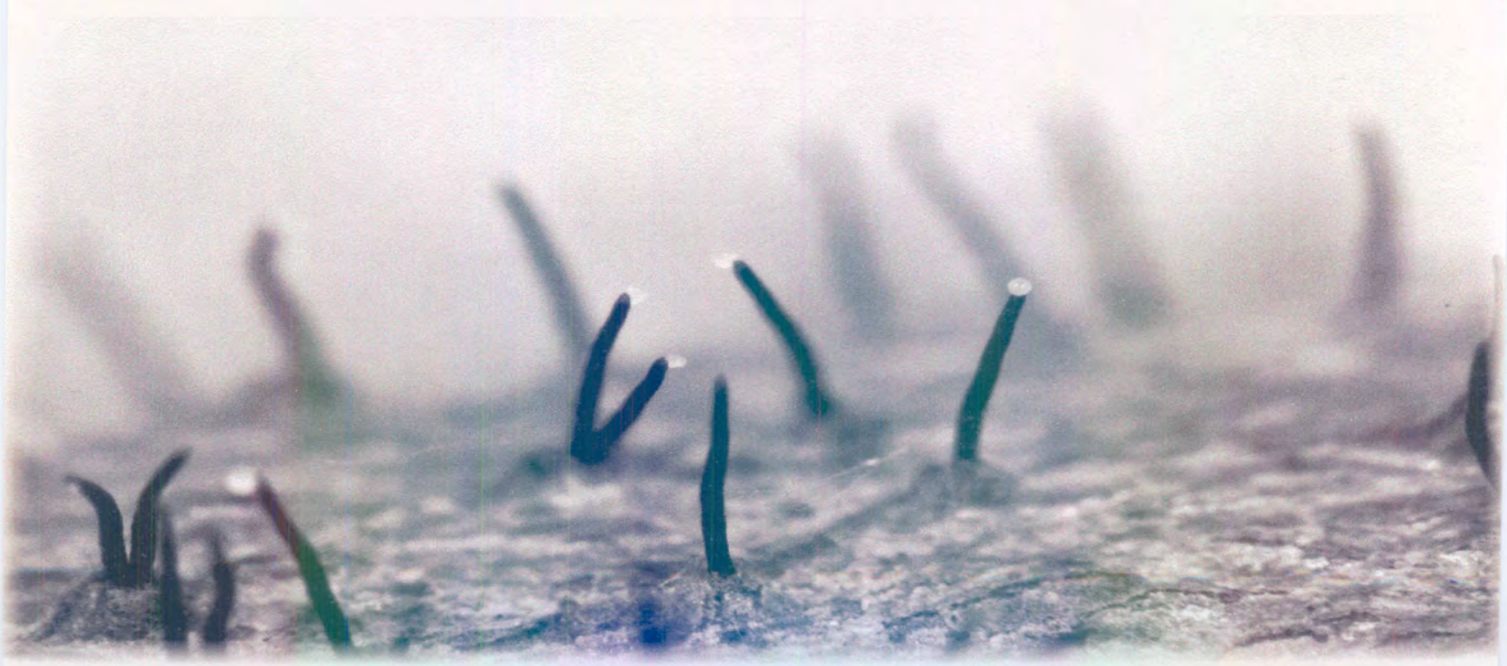
```
[                    570        ]
[                    .          ]

AF046900Br     GCTCTTGCCG -TAAA-C    [479]
AF046896In     ..C.....  -....A.    [482]
AF046893Au     ..........-....A.    [482]
AF046890Ch     ..........-....A.    [482]
AF046894Au     ..........-....A.    [481]
AF046895Au     ..........-....A.    [483]
AF046899Th     .........  G....A.    [483]
AF046891Br     ..........-....A.    [481]
AF046897Ve     ..........-....A.    [481]
AF046892SA     ..........-....A.    [481]
3B3.7__CRY     ..........-....A.    [483]
3B21.3_CRY     ..........-....A.    [483]
3B19.3_CRY     ..........-....A.    [483]
3B4.3__CRY     ..........-....A.    [481]
AF046903Cp     ..........-....A.    [525]
AF046902Cp     ..........-....A.    [529]
AF046901Cp     ..........-....A.    [528]
2498_D.amb     ..-..-..G. T....-.   [463]
```

Figure 2. A phylogenetic tree generated after a heuristic search of the ITS sequence data set. Branch lengths are indicated on top of each branch, while bootstrap values are indicated below. The origin of each isolate is indicated after the Genbank accession number. Clade 1 includes South American isolates, clade 2 represents isolates from South East Asia, and *C. parasitica* isolates are included in clade 3.


Tree length = 261
CI = 0.9847
RI = 0.9861
T characters = 577
T parsimony uninformative = 111
T parsimony informative = 118
AF046900 Brazil
AF046891 Brazil
AF046897 Venezuela
AF046892 South Africa
AF172654 Colombia
AF172655 Colombia
AF172656 Colombia
AF172657 Colombia
AF046896 Indonesia
AF046893 Australia
AF046890 China
AF046894 Australia
AF046895 Australia
AF046899 Thailand
AF046903 C. parasitica
AF046902 C. parasitica
AF046901 C. parasitica
CMW 2498 D.ambigua
51 %
55
100 %
88 %
90 %
65 %
63
100 %
116
10 changes
1
2
3

CHAPTER III

HOMOTHALLISM AND SEXUAL OUTCROSSING IN A COLOMBIAN POPULATION OF *CRYPHONECTRIA CUBENSIS*



HOMOTHALLISM AND SEXUAL OUTCROSSING IN A POPULATION OF *CRYPHONECTRIA CUBENSIS*

ABSTRACT

Cryphonectria cubensis is a well-known pathogen of plantation *Eucalyptus* species in South America, South Africa and South East Asia. In South America, the fungus reproduces sexually through perithecia bearing ascospores, whereas reproduction is predominantly asexual in South Africa. Thus, the diversity of vegetative compatibility groups (VCGs) in South America is greater than that observed in South Africa, suggesting that outcrossing occurs in the former area. The aim of this study was to determine the mating strategy of a Colombian population of *C. cubensis*, and to ascertain whether outcrossing occurs in the fungus. Single ascospore isolates of *C. cubensis* from Colombia were used to inoculate *Eucalyptus* twigs, and allowed to reproduce. Also, progeny from several naturally occurring and laboratory-prepared perithecia, were used in VCG tests and PCR-assisted DNA fingerprinting, in order to detect outcrossing and subsequent recombination of genes. Single ascospore isolates were able to self when inoculated onto *Eucalyptus* twigs. However, three naturally occurring perithecia that displayed recombination at the *vic* loci, were detected out of a sample of 30. PCR fingerprinting profiles also suggested that recombination can occur without affecting the VCG phenotype. The Colombian population of *C. cubensis* is, therefore, predominantly homothallic, but sexual outcrossing occasionally occurs.

INTRODUCTION

Cryphonectria cubensis is an important pathogen of eucalypts (Alfenas *et al.*, 1983; Conradie *et al.*, 1992). The fungus occurs in tropical and sub-tropical areas, including parts of South Africa (Wingfield *et al.*, 1989), South America (Hodges *et al.*, 1979; Van Zyl *et al.*, 1998), South East Asia (Boerboom & Maas, 1970; Sharma *et al.*, 1985), as well as in Australia (Davison & Coates, 1991). On susceptible trees, it causes cankers that can girdle stems, restricting water and nutrient transport (Alfenas *et al.*, 1983; Florence *et al.*, 1986; Hodges *et al.*, 1979; Wingfield *et al.*, 1989), which usually results in tree death.

The disease symptoms and biology of *C. cubensis* vary in different parts of the world. Basal cankers can be found on *Eucalyptus* stems in South Africa where young trees die rapidly (Conradie *et al.*, 1990; Wingfield *et al.*, 1989). In South America, cankers occur higher up on the stems, and bases of older infected trees are typically swollen (Hodges *et al.*, 1979). In Australia, *C. cubensis* has been isolated from the roots of *Eucalyptus* trees that grow in the Mediterranean Western part of that country (Davison & Coates, 1991; Myburg *et al.*, 1999). Furthermore, Wingfield *et al.* (1997) noted that while sexual reproduction occurs abundantly on infected trees in South America, it is absent in South Africa.

Cryphonectria cubensis reproduces sexually through the production of ascospores in perithecia (Hodges *et al.*, 1979) and is believed to be homothallic (Hodges *et al.*, 1979;

Van Heerden, 1999; Van Zyl *et al.*, 1998). Asexual reproduction occurs through the production of pycnidia containing mitospores (Hodges *et al.*, 1979). Populations of the fungus are consequently expected to display low levels of diversity, due to the lack of outcrossing.

Outcrossing in fungal species can be detected using various techniques. In the case of most heterothallic fungi such as *Cryphonectria parasitica*, the presence of sexual fruiting structures is an indication of outcrossing (Milgroom *et al.*, 1993). Sexual recombination also implies that an increase in population diversity, specifically multilocus genotypic variation, might be expected (Milgroom, 1996). Using molecular techniques such as restriction fragment length polymorphisms (RFLPs) (Correll *et al.*, 1992; Milgroom *et al.*, 1993) and randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), recombination can be detected unequivocally (Milgroom, 1996; Milgroom *et al.*, 1992; Milgroom, *et al.*, 1993). The sensitivity of these techniques makes it possible to detect recombination of alleles at specific loci that are scattered throughout the genome. This approach allows for the exclusion of strong selective pressures that may affect characters such as virulence, fungicide sensitivity, and vegetative incompatibility (McDonald & McDermott, 1993).

Thus, the aim of this study was firstly to determine whether single ascospore isolates of *C. cubensis* originating in Colombia, are able to self under laboratory conditions. Isolates of *C. cubensis* from Colombia have not been examined in detail previously. Secondly, the possibility of outcrossing in the Colombian population was assessed by DNA

fingerprinting of progeny from natural and artificial sexual events. Both of these questions were addressed by evaluating VCG diversity within perithecia, and inter-short sequence repeat (ISSR) DNA fingerprinting of progeny from selected perithecia.

MATERIALS AND METHODS

Fungal cultures

Bark cankers caused by *C. cubensis* were from a single plantation near Cali, Colombia. Thirty perithecia, each occurring on an individual tree, were randomly sampled by cross-sectioning the perithecial stromata with a sterile scalpel blade. The pale yellow ascospore masses were suspended in two drops of sterile distilled water (dH_2O). Spore suspensions were examined under the light microscope to ascertain their integrity and presence. A sterile platinum loop was used to streak the ascospore suspension onto the surface of malt extract agar (MEA; 20 g/L malt extract, 20 g/L agar) plates (9 cm diameter). The MEA plates were incubated at 26°C for 16 h, after which they were examined for germination using a dissection microscope. Twelve single germinated spores were removed from each plate, and transferred to fresh MEA plates. The resulting single ascospore cultures were incubated for 1 wk at 26°C, exposed to alternating cycles of light (14 h) and dark (10 h). All isolates were labelled and deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Test for homothallism

Five randomly chosen single ascospore cultures from different trees were used to determine whether *C. cubensis* is homothallic. The five selected cultures were transferred to fresh MEA plates, followed by incubation at 26°C in the dark for 2 days. Mycelial plugs of 5 mm^2 were removed from the actively growing edges of the five resulting colonies, and inoculated into wounds (5 mm^2), made with a sterile scalpel blade on fresh *Eucalyptus grandis* and the hybrid *E. grandis* X *E. camaldulensis* twigs. The twigs were approximately 7 mm in diameter, and the bark was 2 mm thick. Twigs were surface sterilised using 70% (v/v) ethanol, and the ends sealed with molten candle wax. Each isolate was inoculated singly on a twig by placing an MEA plug into the wound, with the mycelium side facing inwards. Wounds were covered with masking tape, and each twig was placed in a clean Petri dish lined with moist, sterile filter paper, followed by incubation for 6 wk at 26°C with alternating cycles of light (14 h) and dark (10 h). Once perithecia were observed, two were randomly sampled and 12 single ascospore isolates were made for each of these perithecia. These were then included in the list of isolates (Table 1) for further study.

Vegetative compatibility tests

VCG tests were carried out to determine whether sexual outcrossing had occurred during the process of ascosporogenesis. An initial test was performed on 10 randomly selected isolates on both oatmeal agar (OMA) and bromocresol green malt extract agar (BGMEA) (Powell, 1995) to determine the sensitivity of the two media. The twelve isolates

resulting from each of 30 perithecia were paired against each other in all possible combinations on BGMEA. Small mycelial plugs (1-2 mm^2) were removed from the edges of actively growing, 6-day old cultures. Each plug was placed mycelium-side down, and *ca.* 4-5 mm from the other isolate, on BGMEA plates. The paired isolates were incubated at 26°C in the dark for 3 days and 4 days to provide two independent scores. Inter-mycelium reactions were scored based on the formation of a barrage line (scored as "-") or the lack thereof (scored as "+"). All experiments were repeated once, resulting in a total of four scores for each combination of isolates.

ISSR-PCR fingerprinting

In order to detect molecular variation between full-sib isolates derived from perithecia that showed variation amongst ascospore cultures at the VCG level, ISSR-PCR DNA fingerprinting was used. DNA was extracted using a modification of the protocol described by Raeder & Broda (1985). Cultures were transferred to 9 cm diameter MEA plates covered with sterile cellophane squares (5 cm^2, one square per plate), and incubated at 26°C in the dark for 5 days. Cellophane squares bearing mycelium were removed from the Petri dishes and immediately ground to a fine powder in the presence of liquid nitrogen, using a ceramic mortar and pestle. The powder was transferred to sterile 1·5 ml microcentrifuge tubes and immediately supplemented with 200 µl extraction buffer (200 mM Tris-Cl pH 8·5; 250 NaCl; 25 mM EDTA, 0·5% w/v SDS). Mycelium was homogenised in extraction buffer by vortexing the tubes for 10 s. The homogenate was incubated in a 65°C water bath for 30 min before addition of a further

200 µl extraction buffer. This mixture was subsequently vortexed for 5 s. The mycelial suspensions were repeatedly extracted with phenol-chloroform (1:1, v/v) until a clean interphase was obtained. Supernatant was transferred to clean tubes and DNA precipitated overnight with the addition of 0·1 vol 3 M Na-Ac (pH 5·2) and 0·6 vol ice cold isopropanol. DNA was pelleted by centrifugation at 15 000 x *g* for 30 min, and the pellets were washed twice with 70% v/v ethanol. The DNA was dried for 10 min at 45°C using an Eppendorf Concentrator 5301 (Eppendorf-Netheler-Hinz, GmbH), redissolved in 50 µl dH_2O, and stored at –20°C for further experimentation.

One microliter of extracted DNA (~25 ng) was used in an inter-short sequence repeat (ISSR) (Hantula *et al.*, 1996) polymerase chain reaction (PCR) with each of two primers: ACA (BDB$\{ACA\}_5$) or GT (HV$\{GT\}_5$). Each PCR reaction contained 5 µl 10x PCR Reaction Buffer, 25 mM $MgCl_2$, 200 µM of each dNTP, 2 µM of either the ACA or GT primer, 5U of SuperTherm *Taq* polymerase (Southern Cross Biotechnology, South Africa), and 1 µl of DNA solution. Sterile distilled water was added to a final volume of 50 µl and overlaid with mineral oil to prevent evaporation during thermal cycling. PCR was performed on a Hybaid OmniGene (Hybaid, U.K.) thermal cycler and included 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C (GT primer) or 47°C (ACA primer) for 1 min, and DNA synthesis at 72°C for 1 min. The protocol was followed by a final extension at 72°C for 6 min. DNA amplification was assessed by electrophoresis of 10 µl PCR reaction mix on a 1% w/v agarose gel at 6 V/cm for 1 h.

ISSR-PCR fingerprints were obtained by electrophoresis of 10 µl PCR product on 6% w/v native polyacrylamide gels at 9·3 V/cm for 6 h. A 100 bp DNA ladder (Promega, USA) was used as a molecular weight standard on all gels. Gels were silver stained using the protocol described by Bassam & Cactano-Annolés (1993), photographed and dried for future reference.

Genotyping and data analysis

Multilocus haplotypes were scored as genotypes by comparing the presence or absence of bands between samples. Non-reproducible bands were omitted from the analyses. The clonal fraction within each perithecium was determined by dividing the number of observed clones with the sample size, hence

$$Cf = \frac{[N-(C-1)]}{N}$$

where N is the sample size and C is the number of distinct genotypes within each perithecium. The number of clones (C) must be corrected by the subtraction of one, in order to allow for a clonal fraction of 100%, which would indicate self-fertilisation with no outcrossing.

RESULTS

Fungal cultures

Cultures of *C. cubensis* were isolated with relative ease from infected *Eucalyptus* material collected in Colombia. About 30% of all ascospores germinated (Figure 1A-B). Even though isolation was inefficient, single ascospore cultures were readily obtained for each of the sampled perithecia.

Test for homothallism

Of the five isolates tested, only one (Per3a10) produced perithecia profusely when inoculated onto a *Eucalyptus* twig (Figure 1C-F). The perithecia produced by this isolate possessed necks *ca.* 3 mm long, and these contained well-developed ascospore masses that could be used for single ascospore isolations. Three other isolates, Per2a9, Per22a6 and Per27a4, produced fewer perithecia with much longer necks (up to 7 mm). Perithecia produced by these isolates were either empty, or ascospores were produced in very low number. These ascospores also did not germinate when single ascospore isolations were attempted. One isolate, Per15a6, did not produce perithecia.

Vegetative compatibility tests

An initial test of the sensitivity of two media to descriminate VCGs, revealed that weak barrages could be detected on BGMEA while these were undetectable on OMA. VCG tests on 12 isolates from each of the 30 perithecia using BGMEA, made it possible to discriminate between isolates that belonged to different VCGs. Of the 30 perithecia tested, 27 contained ascospores belonging to a single VCG. Ascospores from the remaining three perithecia (Per7, Per15, and Per23) belonged to more than one VCG (Figures 2 & 3).

ISSR-PCR fingerprinting

PCR amplification of inter-short sequence repeats (ISSRs) yielded products between 200 bp and 5000 bp long. Only products between 400 and 1500 bp could be consistently amplified several times with varying PCR conditions, and were thus considered reproducible. These were subsequently identified as the partial fingerprint profiles to be used for assessment of multilocus haplotypes (genotypes) (Figure 4).

Genotyping and data analysis

A wide variety of ISSR genotypes were detected in all three perithecia that showed VCG variation in ascospore progeny (Figure 4). The average clonal fractions (Table 2) present in perithecia Per7, Per15 and Per23 were 67%, 63% and 50%, respectively. Single

ascospore isolates originating from perithecium Per3, which displayed no VCG variation, also showed variation in their ISSR genotypes (Figure 4A). The clonal fraction of progeny from this perithecium was 71%.

DISCUSSION

This study has shown that *C. cubensis* from Colombia is homothallic. The finding is supported by the fact that single ascospore isolates are capable of completing the sexual cycle and producing viable ascospores, in the absence of other isolates. Also, the progeny from these perithecia belonged to one VCG, and these were genetically identical based on ISSR DNA fingerprints. A similar result was found by Van Heerden (1999) when single ascospore isolates of *C. cubensis* from Colombia were inoculated onto *Eucalyptus* twigs, but progeny from these self-fertilisations were not analysed at the genetic level. The fungus was originally described as homothallic (Hodges *et al.*, 1979), but the high level of VCG diversity observed in South American populations (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998) led us to question this observation.

The fact that three Colombian *C. cubensis* isolates (Per2a9, Per22a6, and Per27a4) produced barren perithecia or perithecia containing very few infertile ascospores, when singly incolated onto *Eucalyptus* twigs, suggests that a self-incompatibility or self-sterility system may function in this fungus. A unidirectional mating type switching system was described for the ascomycete, *Ceratocystis coerulescens* (Harrington & McNew, 1995; Harrington & McNew, 1997), where self-fertilised perithecia produce

both self-fertile (homothallic) and self-sterile (heterothallic) ascospore progeny. Although the exact genetic system responsible for mating-type switching in *C. coerulescens* has not been elucidated, it is known that a segment of DNA is lost from the *MAT-2* locus (Witthuhn *et al.*, 1999). No information on the mating-type loci of *C. cubensis* is currently available, which makes it difficult to speculate on the genetic system underlying sexual self-incompatibility in this species.

Using vegetative incompatibility tests (VCGs), it was possible to show that outcrossing occurs in the Colombian population of *C. cubensis* investigated in this study. However, only three of 30 perithecia contained progeny belonging to more than one VCG, indicating that the parents of the cross belonged to different VCGs. The progeny from these outcrossing events could not be unequivocally assigned to single VCGs (Figure 3). We hypothesise that the discrepancies in VCG assignments were encountered due to recombination of genes controlling vegetative incompatibility, and thus the subsequent emergence of isolates representing intermediate forms between well-defined VCGs. A similar phenomenon was observed by Cortesi *et al.* (1996) when sub-populations of *C. parasitica* from Italy were analysed for VCG diversity.

The parent strains of the *C. cubensis* progeny resulting from outcrossing events were not closely related, since ISSR-PCR DNA fingerprinting profiles of these progeny differed markedly. However, the parents of the 27 perithecia containing progeny belonging to a single VCG were either genetically identical, or very closely related, based on fingerprint profiles. Therefore, it seems that outcrossing between unrelated individuals is a rare event

in the Colombian *C. cubensis* population, and that it occurs at a frequency of *ca.* 10%. We consequently expect that self-fertilisation (Glass & Kuldau, 1992) or biparental inbreeding (Milgroom, 1995) are the main modes of sexual reproduction in *C. cubensis* from Colombia.

DNA fingerprints from 12 single ascospore isolates from a perithecium that contained progeny belonging to a single VCG, yielded a clonal fraction which was lower than that expected for a true self (Table 2). This result can only be explained by the fact that the parents of this outcrossing event must have been closely related or belonged to the same VCG. This would then have given rise to a limited, but detectable amount of recombination in the progeny. Biparental inbreeding (Francis & St Clair, 1993; Milgroom *et al.*, 1993), therefore, occurs in the population, but the extent of this form of sexual reproduction remains unclear.

Results of this study have demonstrated that *C. cubensis* from Colombia is homothallic but that it is capable of outcrossing. The same reproductive strategy has been described for other fungi in the genera *Phytophthora* and *Pythium* (Francis & St Clair, 1993; Whisson *et al.*, 1994). The results also suggest that outcrossing in homothallic species, also referred to as relative homothallism (Beraha & Garber, 1985), is not rare in nature. Relative homothallism requires further investigation in this population of *C. cubensis*.

The *C. cubensis* population from Colombia displays a mixed mating system, where it is possible for strains to undergo self-fertilisation, or outcross with either closely related or

distantly related individuals. A similar situation was described for a population of *C. parasitica* by Milgroom *et al.* (1993). These authors also estimated the outcrossing rate in the population, using neutral genetic markers. Similar markers are not currently available for *C. cubensis*. Future efforts should, therefore, focus on the development of DNA markers for population studies of this fungus.

6. REFERENCES

Alfenas, A. C., Jeng, R. & Hubbes, M. (1983). Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**, 197-205.

Bassam, B. J. & Cactano-Anollés, G. (1993). Silver staining of DNA in polyacrylamide gels. *Applied Biochemistry and Biotechnology* **42**, 181-188.

Beraha, L. & Garber, E. D. (1985). Relative heterothallism and production of hybrid perithecia by auxotrophic mutants of *Glomerella cingulata* from apple. *Phytopathologische Zeitschrift* **112**, 32-39.

Boerboom, J. H. A. & Maas, P. W. T. (1970). Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**, 94-99.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990). Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**, 43-49.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1992). Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. *European Journal of Forest Pathology* **22**, 312-315.

Correll, J. C., Gordon, T. R. & McCain, A. H. (1992). Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Phytopathology* **82**, 415-420.

Cortesi, P., Milgroom, M. G. & Bisiach, M. (1996). Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. *Mycological Research* **100**, 1087-1093.

Davison, E. M. & Coates, D. J. (1991). Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157-160.

Florence, E. J. M., Sharma, J. K. & Mohanan, C. (1986). A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384-386.

Francis, D. M. & St Clair, D. A. (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. *Current Genetics* **24**, 100-106.

Glass, N. L. & Kuldau, G. A. (1992). Mating type and vegetative incompatibility in filamentous Ascomycetes. *Annual Review of Phytopathology* **30**, 201-224.

Hantula, M., Dusabenyagasani, M. & Hamelin, R. C. (1996). Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* **26**, 159-166.

Harrington, T. C. & McNew, D. L. (1995). Mating type switching and self-fertility in *Ceratocystis*. *MSA Abstracts* 18.

Harrington, T. C. & McNew, D. L. (1997). Self-fertility and uni-directional mating-type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**, 52-59.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

McDonald, B. A. & McDermott, J. M. (1993). Population genetics of plant pathogenic fungi. *BioScience* **43**, 311-319.

Milgroom, M. G. (1995). Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany* **73(Suppl. 1)**, S311-S319.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y. (1993). Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **70**, 385-392.

Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992). DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **131**, 297-306.

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

Powell, W. A. (1995). Vegetative incompatibility and mycelial death of *Cryphonectria parasitica* detected with a pH indicator. *Mycologia* **87**, 738-741.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**, 17-20.

Sharma, J. K., Mohanan, C. & Florence, E. J. M. (1985). Occurrence of Cryphonectria canker disease of *Eucalyptus* in Kerala, India. *Annals of Applied Biology* **106**, 265-276.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis.*

Van Heerden, S. W., Wingfield, M. J., Coutinho, T. & Van Zyl, L. M. (1997). Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv. (Abstract).

Van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998). Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**, 41-47.

Whisson, S. C., Drenth, A., Maclean, D. J. & Irwin, J. A. (1994). Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Current Genetics* **27**, 77-82.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Wingfield, M. J., Van Zyl, L. M., Van Heerden, S., Myburg, H. & Wingfield, B. D. (1997). Virulence and the genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In *Physiology and genetics of tree-phytophage interactions*, pp. 163-172. Edited by F. Lieutier, W. J. Mattson & M. R. Wagner. Gujan, France: INRA Editions.

Witthuhn, R. C., Harrington, T. C., Wingfield, B. D. & Wingfield, M. J. (1999). Deletion of the *MAT-2* idiomorph during uni-directional mating type switching in *Ceratocystis*. *Current Genetics* (*submitted*).

Table 1. Isolates of Colombian *C. cubensis* used in this study.[a]

Perithecium Number[b]	Isolate Numbers[c]	Number of VCGs[d]
Per1	Per1a1 to Per1a12	1
Per2†	Per2a1 to Per2a12	1
Per3*†	Per3a1 to Per3a12	1
Per4	Per4a1 to Per4a12	1
Per5	Per5a1 to Per5a12	1
Per6	Per6a1 to Per6a12	1
Per7*	Per7a1 to Per7a12	>1
Per8	Per8a1 to Per8a12	1
Per9	Per9a1 to Per9a12	1
Per10	Per10a1 to Per10a12	1
Per11	Per11a1 to Per11a12	1
Per12	Per12a1 to Per12a12	1
Per13	Per13a1 to Per13a12	1
Per14	Per14a1 to Per14a12	1
Per15*†	Per15a1 to Per15a12	>1
Per16	Per16a1 to Per16a12	1
Per17	Per17a1 to Per17a12	1
Per18	Per18a1 to Per18a12	1
Per19	Per19a1 to Per19a12	1
Per20	Per20a1 to Per20a12	1
Per21	Per21a1 to Per21a12	1
Per22†	Per22a1 to Per22a12	1
Per23*	Per23a1 to Per23a12	>1
Per24	Per24a1 to Per24a12	1
Per25	Per25a1 to Per25a12	1
Per26	Per26a1 to Per26a12	1
Per27†	Per27a1 to Per27a12	1
Per28	Per28a1 to Per28a12	1
Per29	Per29a1 to Per29a12	1
Per30	Per30a1 to Per30a12	1
Self1*‡	Self1a1 to Self1a12	1
Self2*‡	Self2a1 to Self2a12	1

[a] All isolates were kept on malt extract agar in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[b] Perithecia followed by a * are those included in DNA fingerprinting analyses; those followed by † were used for inoculation of *Eucalyptus* twigs and those followed by ‡ resulted from a controlled laboratory induction of sexual reproduction.

[c] Isolate numbers (Per) are those for each of 30 perithecia from which 12 single ascospore cultures were collected. Those labelled "Self" are the result of self-fertilisation events on *Eucalyptus* twigs.

[d] The number of VCGs are indicated as "1" when all isolates from a perithecium belonged in the same VCG, and ">1" when incompatibility between single ascospore isolates from within a perithecium was present.

Table 2. Multilocus haplotypes (genotypes) and average clonal fraction for each of the four perithecia analysed with ISSR DNA fingerprinting.

Perithecium Number[a]	Number of multilocus haplotypes		Average Clonal Fraction[b]
	Primer ACA	Primer GT	
Per3	4 (75%)	5 (67%)	71%
Per7	6 (58%)	4 (75%)	67%
Per15	6 (58%)	5 (67%)	63%
Per23	8 (42%)	6 (58%)	50%
Self1[1]	1 (100%)	1 (100%)	100%
Self2[1]	1 (100%)	1 (100%)	100%

[a] Perithecia resulting from controlled induction of sexual reproduction in isolate Per3a10.
[b] The average clonal fraction was calculated by averaging the *Cf* observed within each perithecium for both the primers, ACA and GT. Values in brackets are the calculated *Cf* for each primer applied to each perithecium.

Figure 1. Morphological structures associated with *Cryphonectria cubensis* in Colombia. (A) Ascus containing eight two-celled ascospores (septa not visible). (B) Germinating ascospores after incubation in sterile distilled water for 5 hours. One or both of the cells of each ascospore can form a germination tube. (C) Perithecia occur in clusters of two or more, but single perithecia can also be observed. (D) Point of inoculation on a *Eucalyptus* twig. Mature perithecia (arrows) can be observed around the wound. (E) Five-day old perithecia starting to exude ascospores (arrows) from their necks. (F) Young perithecium developing on an inoculated *Eucalyptus* twig after a six week incubation period. Perithecium maturation progresses for a further 3-5 days before viable ascospores are produced.

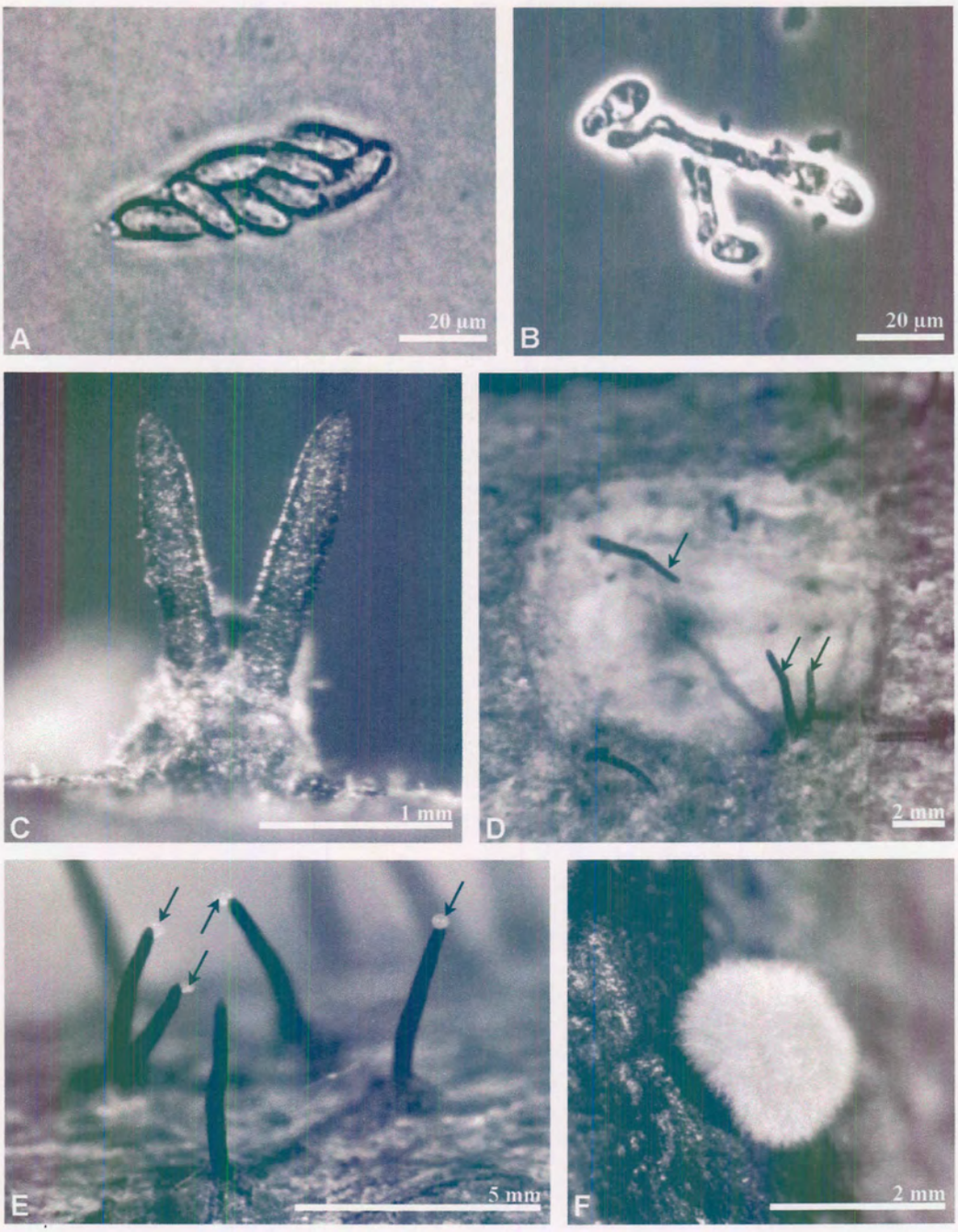


Figure 2. Vegetative incompatibility amongst 12 single ascospore isolates derived from perithecium Per7. All 12 single ascospore isolates were paired in all possible combinations. Isolate numbers are indicated at the positions where they were placed on the plates. Incompatibility is seen as a dark line of barrage formation at the contact line between two incompatible isolates, and compatibility as confluent mycelium between two isolates.

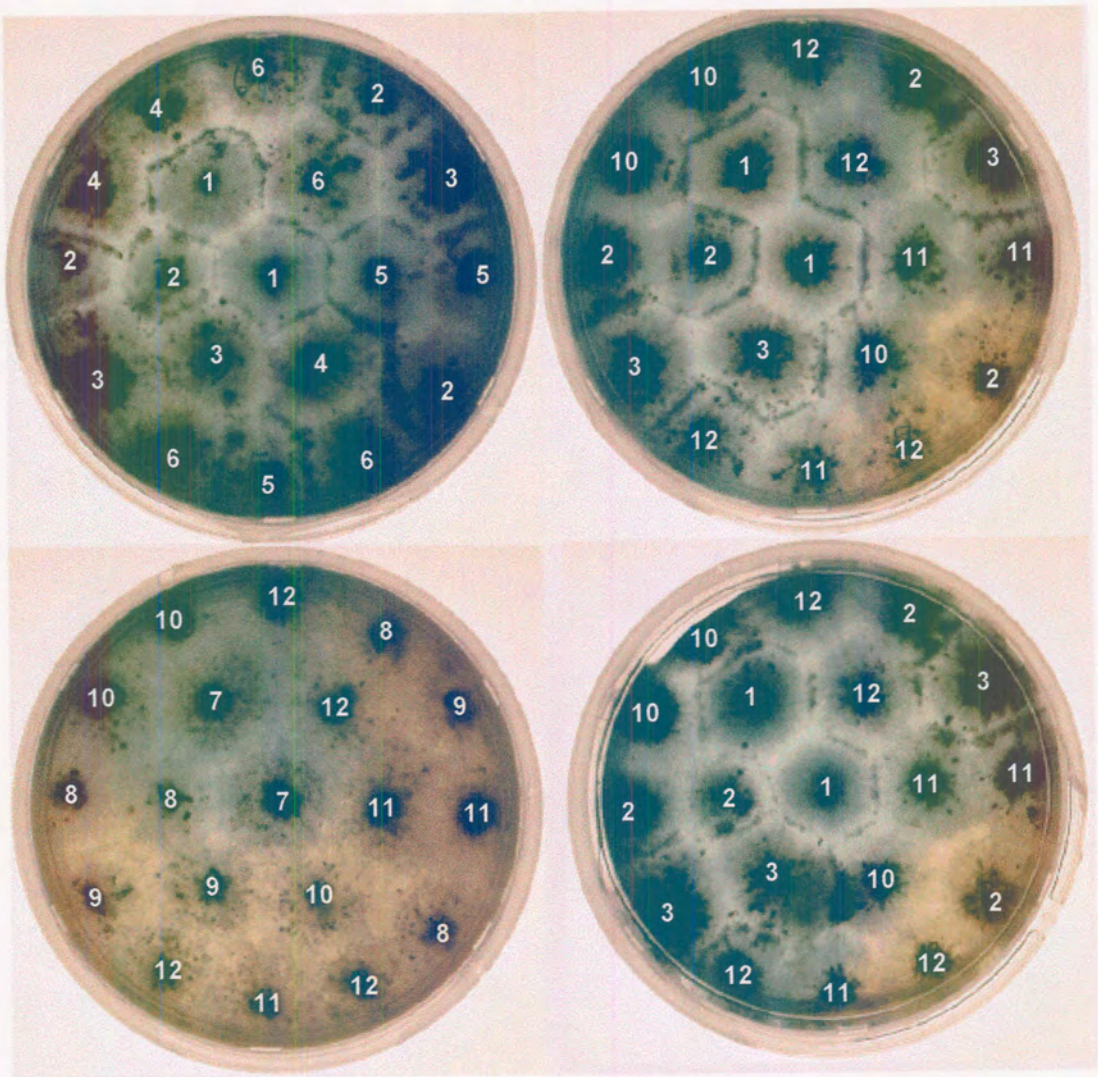
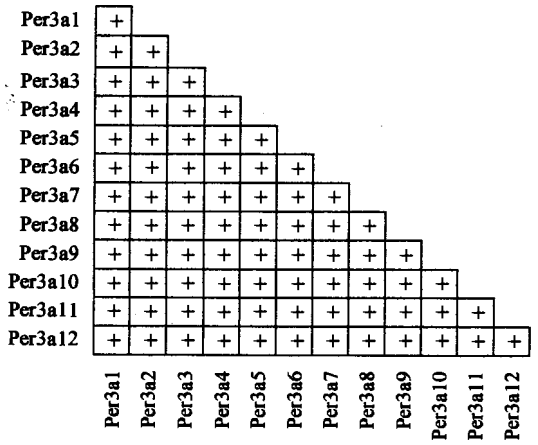
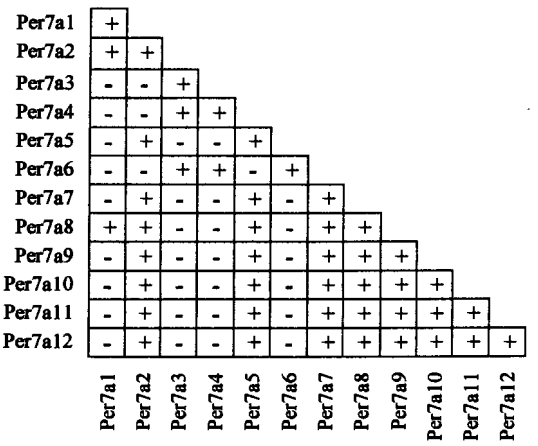

6 2 4 4 1 6 3 2 2 1 5 5 3 3 4 2 6 5 6
12 10 2 10 1 12 3 2 2 1 11 11 3 3 10 2 12 11 12
12 10 8 10 7 12 9 8 8 7 11 11 9 9 10 8 12 11 12
12 2 10 1 12 3 10 2 1 11 11 3 10 2 3 12 11 12

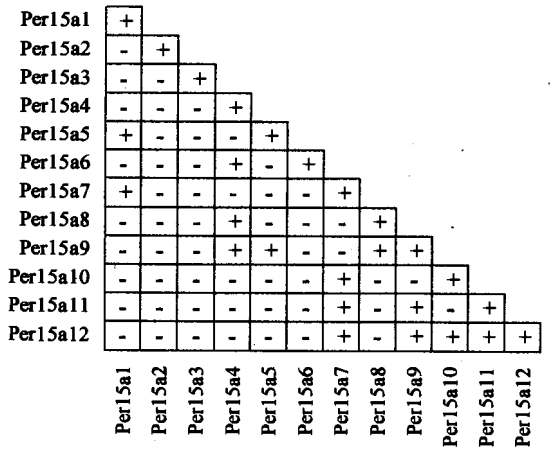
Figure 3. VCG reactions seen in isolates from perithecia Per3, Per7, Per15 and Per23. Barrage formation is indicated as "-", while no interaction is indicated as "+".



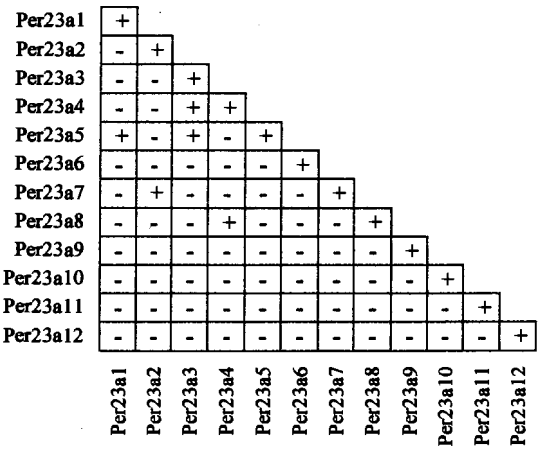
	Per3a1	Per3a2	Per3a3	Per3a4	Per3a5	Per3a6	Per3a7	Per3a8	Per3a9	Per3a10	Per3a11	Per3a12
Per3a1	+											
Per3a2	+	+										
Per3a3	+	+	+									
Per3a4	+	+	+	+								
Per3a5	+	+	+	+	+							
Per3a6	+	+	+	+	+	+						
Per3a7	+	+	+	+	+	+	+					
Per3a8	+	+	+	+	+	+	+	+				
Per3a9	+	+	+	+	+	+	+	+	+			
Per3a10	+	+	+	+	+	+	+	+	+	+		
Per3a11	+	+	+	+	+	+	+	+	+	+	+	
Per3a12	+	+	+	+	+	+	+	+	+	+	+	+



	Per7a1	Per7a2	Per7a3	Per7a4	Per7a5	Per7a6	Per7a7	Per7a8	Per7a9	Per7a10	Per7a11	Per7a12
Per7a1	+											
Per7a2	+	+										
Per7a3	-	-	+									
Per7a4	-	-	+	+								
Per7a5	-	+	-	-	+							
Per7a6	-	-	+	+	-	+						
Per7a7	-	+	-	-	+	-	+					
Per7a8	+	+	-	-	+	-	+	+				
Per7a9	-	+	-	-	+	-	+	+	+			
Per7a10	-	+	-	-	+	-	+	+	+	+		
Per7a11	-	+	-	-	+	-	+	+	+	+	+	
Per7a12	-	+	-	-	+	-	+	+	+	+	+	+



	Per15a1	Per15a2	Per15a3	Per15a4	Per15a5	Per15a6	Per15a7	Per15a8	Per15a9	Per15a10	Per15a11	Per15a12
Per15a1	+											
Per15a2	-	+										
Per15a3	-	-	+									
Per15a4	-	-	-	+								
Per15a5	+	-	-	-	+							
Per15a6	-	-	-	+	-	+						
Per15a7	+	-	-	-	-	-	+					
Per15a8	-	-	-	+	-	-	-	+				
Per15a9	-	-	-	+	+	-	-	+	+			
Per15a10	-	-	-	-	-	-	+	-	-	+		
Per15a11	-	-	-	-	-	-	+	-	+	-	+	
Per15a12	-	-	-	-	-	-	+	-	+	+	+	+

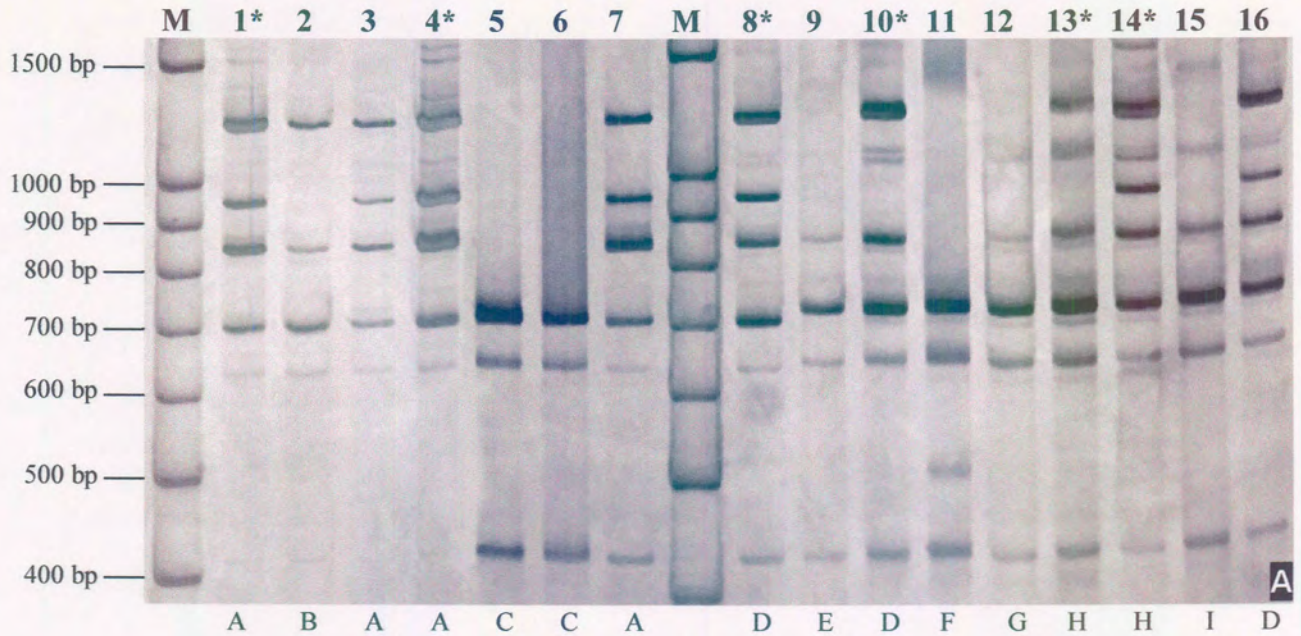


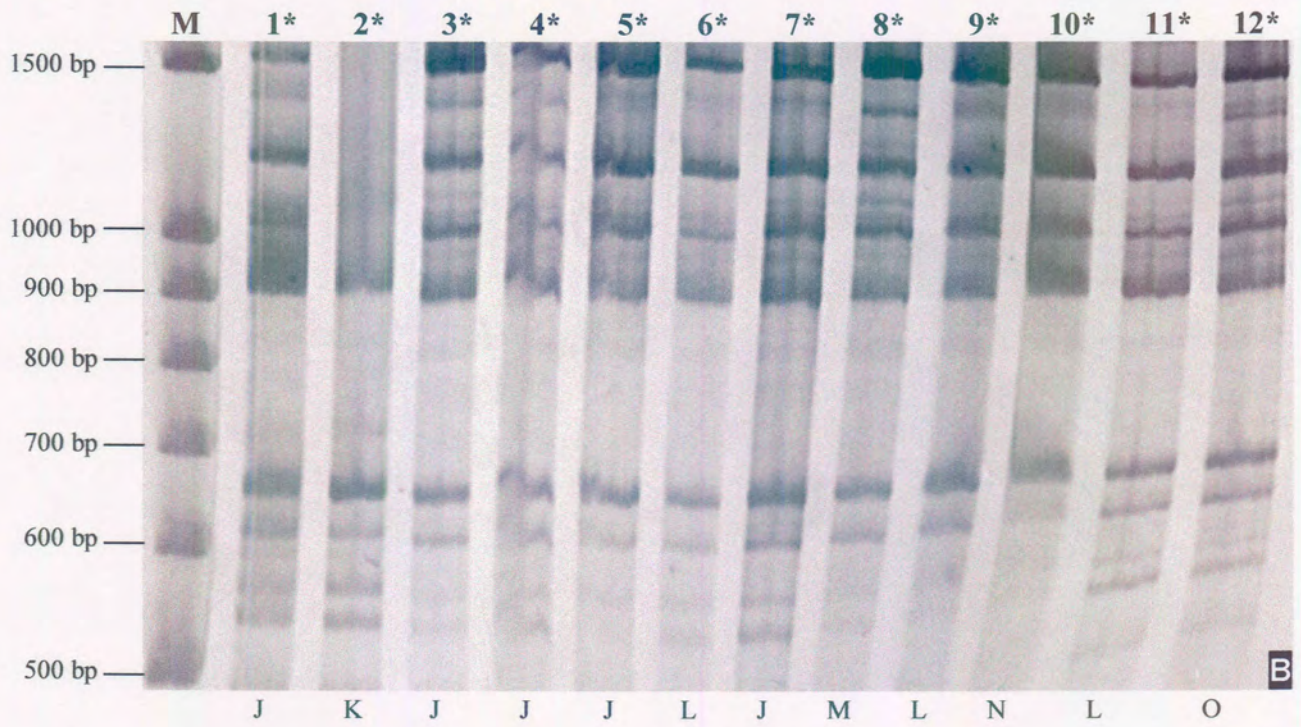
	Per23a1	Per23a2	Per23a3	Per23a4	Per23a5	Per23a6	Per23a7	Per23a8	Per23a9	Per23a10	Per23a11	Per23a12
Per23a1	+											
Per23a2	-	+										
Per23a3	-	-	+									
Per23a4	-	-	+	+								
Per23a5	+	-	+	-	+							
Per23a6	-	-	-	-	-	+						
Per23a7	-	+	-	-	-	-	+					
Per23a8	-	-	-	+	-	-	-	+				
Per23a9	-	-	-	-	-	-	-	-	+			
Per23a10	-	-	-	-	-	-	-	-	-	+		
Per23a11	-	-	-	-	-	-	-	-	-	-	+	
Per23a12	-	-	-	-	-	-	-	-	-	-	-	+

Figures 4 A & B. Representative polyacrylamide gels showing multilocus haplotypes of several full-sib progeny from three separate perithecia. Lane numbers are indicated at the top of each image, while genotype designations are indicated at the bottom. An asterisk (*) with a lane number indicates that bands migrating slower than 1500 bp were present.

(A) Lanes marked with M, 100 bp Molecular Weight Marker; Lanes 1-7, Multilocus haplotypes of seven single ascospore isolates resulting from perithecium Per3. Lanes 8-16, Multilocus haplotypes of nine single ascospore isolates from perithecium Per7.

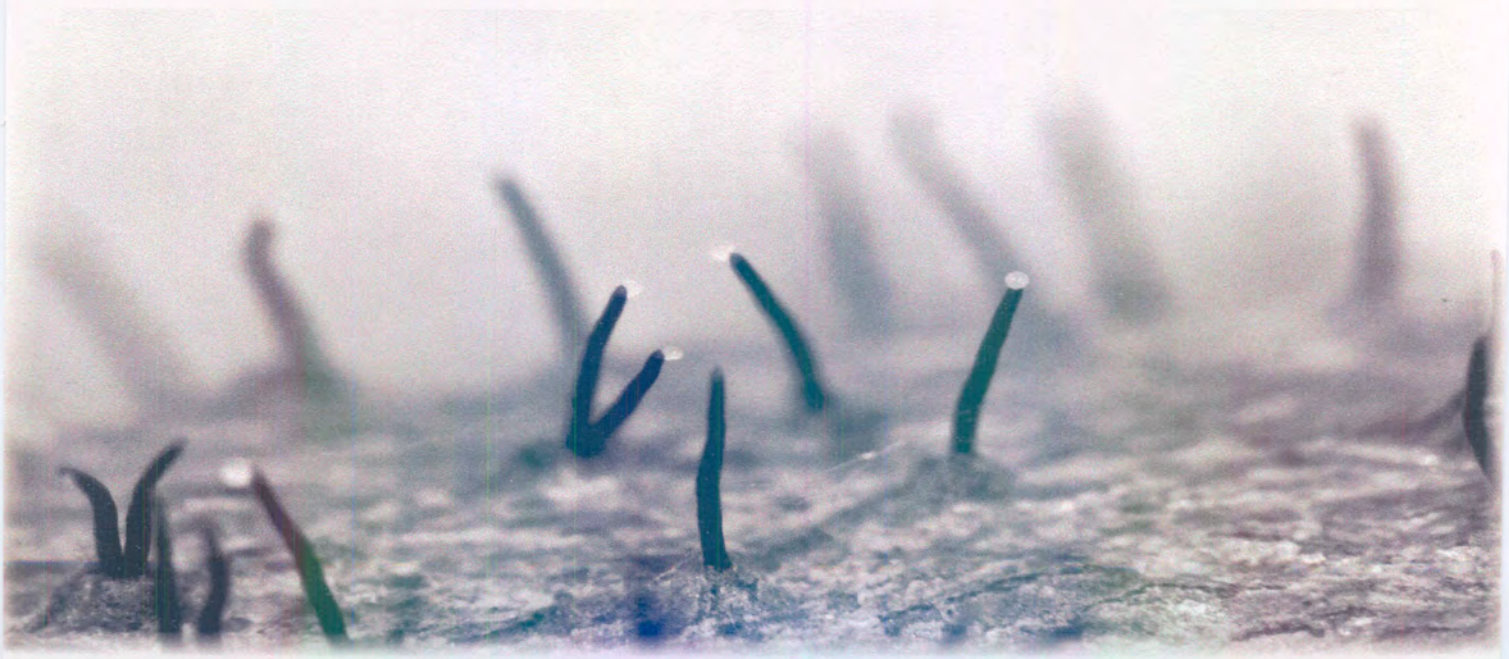
(B) Lane M, 100 bp Molecular Weight Marker; Lanes 1-12, Multilocus haplotypes of twelve single ascospore isolates from perithecium Per15.





CHAPTER IV

GENOTYPIC DIVERSITY OF A COLOMBIAN POPULATION OF *CRYPHONECTRIA CUBENSIS*



GENOTYPIC DIVERSITY OF A COLOMBIAN POPULATION OF *CRYPHONECTRIA CUBENSIS* BASED ON VCGS AND RAPDS

ABSTRACT

Cryphonectria cubensis is an important fungal pathogen of *Eucalyptus* trees that causes Cryphonectria canker resulting in significant losses to forestry industries worldwide. The genetic diversity in several populations of the fungus, mainly from South America, has previously been investigated. Data obtained from these studies are important in tree breeding strategies. Furthermore, the feasibility of using hypovirulence as a biocontrol strategy is directly linked to the diversity of the target population. The aim of this study was to determine the diversity of a Colombian population of *C. cubensis*. Diversity of this population was determined using vegetative compatibility group (VCG) analyses and randomly amplified polymorphic DNA (RAPD) fingerprinting. Genotypic diversity, based on the VCG phenotype, was 37% in this population. Using RAPD data, however, a genotypic diversity of 100% was observed, since each isolate was unique based on RAPD fingerprints. The diversity of the population is therefore high, and biocontrol through hypovirulence is unlikely to succeed.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is an ascomycetous pathogen of plantation eucalypts in the tropics and sub-tropics of the world (Conradie *et al.*, 1992; Florence *et al.*, 1986; Hodges *et al.*, 1979; Hodges, 1980; Wingfield *et al.*, 1989). The fungus causes a stem canker disease, known as Cryphonectria canker, on susceptible *Eucalyptus* trees (Conradie *et al.*, 1990; Conradie *et al.*, 1992; Florence *et al.*, 1986). This disease is cause for concern, due to significant losses experienced by forestry industries, particularly those practising monoculture (Wingfield *et al.*, 1989).

The population diversity of plant pathogenic fungi is an important component of meaningful management programmes. For instance, diversity impacts on the ability to capitalise on hypovirulence-associated biological control (Cortesi *et al.*, 1996; Heiniger & Rigling, 1994; Scibilia & Shain, 1989), as well as the durability of resistance in plants (Melchinger, 1990). Thus, a diverse pathogen population would be able to overcome inbred plant resistance more rapidly, due to the broad genetic base in the pathogen population. This genetic base allows the population to adapt to new conditions (Smith, 1968). Therefore, much effort has been directed towards investigating the diversity and dynamics of important plant pathogen populations (Anagnostakis *et al.*, 1986; Bayman & Cotty, 1991; Brasier, 1987; Burdon & Roelfs, 1985a; Burdon & Roelfs, 1985b; Correll *et al.*, 1992; Milgroom & Lipari, 1995).

Several populations of *C. cubensis* have been studied at the phenotypic level (Alfenas *et al.*, 1984; Davison & Coates, 1991; Van Heerden, 1999; Van Zyl, 1995). Van Heerden (1999) reported on the diversity of the South African population of the

fungus, while a Brazilian population has previously been studied by Van Zyl (1995). A comparison between the South African and South American populations (Van Heerden, 1999) showed that the former population is much less diverse than the latter. Also, the South African population rarely undergoes sexual reproduction (Wingfield *et al.*, 1997). In contrast, sexual reproduction is common in *C. cubensis* in all other areas where the fungus occurs (Florence *et al.*, 1986; Hodges *et al.*, 1979; M.J. Wingfield, *unpublished*).

The chestnut blight pathogen, *Cryphonectria parasitica*, is a close relative of *C. cubensis* (Myburg *et al.*, 1999). Biocontrol of *C. parasitica* through hypovirulence has been effective in Europe, where the VCG diversity is relatively low (Cortesi *et al.*, 1996; Heiniger & Rigling, 1994). However, in Northern America the success of biological control through hypovirulence has been restricted by the high degree of genetic diversity in the *C. parasitica* population (Anagnostakis & Kranz, 1987; Milgroom, 1995). It is, therefore, important to determine the diversity of a target population before hypovirulence is considered as a possible management tool.

Several techniques for determining the population diversity of fungi are available. The most widely used technique is vegetative incompatibility (Anagnostakis, 1982; Anagnostakis & Kranz, 1987; Bégueret *et al.*, 1994; Correll *et al.*, 1992; Leslie, 1993). Two isolates that have the same alleles at their vegetative incompatibility loci, can undergo hyphal fusion (Newhouse & MacDonald, 1991). When allelic differences occur at these loci, an incompatibility reaction is elicited, that results in the formation of a barrage (Anagnostakis, 1982; Beisson-Schecroun, 1962; Bernet, 1965). These isolates are referred to as being in different vegetative compatibility groups (VCGs).

Vegetative incompatibility is genetically controlled, in the case of *C. parasitica* by five loci (Anagnostakis, 1982). The cumulative expression of the *vic* genes present at these loci results in a phenotype that can be tested (Rizwana & Powell, 1995). One difficulty in conducting such analyses is the detection of isolates that represent intermediate forms ("bridges") between VCG "clusters", especially in sexually reproducing populations (Cortesi *et al.*, 1996).

Molecular techniques can be used to circumvent the difficulties encountered in conducting VCG analyses (Correll *et al.*, 1992). In *C. cubensis*, however, no previous studies have been performed to test population diversity at the DNA level. Myburg (1997) used RAPD profiles to investigate the relatedness of *C. cubensis* isolates from various parts of the world, but specific molecular markers to test population diversity have not been developed for this species. Such markers are, however, available for *C. parasitica* (Milgroom *et al.*, 1992; Milgroom *et al.*, 1993) and several other fungi (Francis & St Clair, 1993; Goodwin *et al.*, 1992; Groppe *et al.*, 1995; Whisson *et al.*, 1994). A molecular population diversity study of *C. cubensis* would need to be based on non-specific PCR fingerprinting techniques (Milgroom *et al.*, 1992) in order to detect genotypic differences. The aim of this study was to determine the diversity of a *C. cubensis* population from Colombia, using VCGs and randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) fingerprinting.

MATERIALS AND METHODS

Fungal cultures

Perithecia of *C. cubensis* were randomly sampled from two adjacent plantations of five year old *E. grandis* near Cali, Colombia. One single ascospore isolate was collected from each of 29 perithecia occurring on different trees in one plantation (A) and 30 isolates were collected in a similar fashion from the second plantation (B). The sample size was therefore 59, and represented a random sample from the overall population. All isolates are maintained on 20% (w/v) malt extract agar (MEA) in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Vegetative compatibility groups

All isolates were transferred to fresh MEA plates and grown in the dark at 26°C for three days. A small (4 mm^2) mycelial plug was removed from the actively growing margin of each culture and these were placed 5 mm from each of the other isolates on bromocresol green malt extract agar (BGMEA) (Powell, 1995). All isolates were paired in all possible combinations on this medium. Plates were incubated in the dark at 26°C for three and five days to facilitate two respective scores. The entire experiment was repeated to provide a total of four independent scores for each interaction. When two isolates were in the same VCG, the interaction was scored as "0", while it was scored as "1" when barrage formation was detectable.

RAPD DNA fingerprinting

DNA isolation was performed using a modification of the protocol described by Murray and Thompson (1980). Fungal isolates were inoculated into 0·75 ml liquid malt extract broth (20 g/l) in 1·5 ml microcentrifuge tubes. The tubes were incubated in a horizontal position at 26°C in the dark for three days, after which they were centrifuged at 10 000 x *g* for 10 min to pellet the actively growing mycelium. Growth medium was decanted and 400 µl CTAB extraction buffer (5% w/v CTAB, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; 1·4 M NaCl; 0·2% v/v 2-mercaptoethanol; 20 mM EDTA; 100 mM Tris-HCl pH 8·0; 1% w/v polyvinylpyrrolidone) was added. Mycelium was homogenised using a tight fitting plastic pestle and subsequently incubated at 60°C for 1 h. Phenol-chloroform (1:1) extractions were performed until a clean interphase was obtained. DNA was then precipitated with the addition of 0·1 vol 3M NaAc (pH 5·2) and 0·6 vol ice-cold isopropanol. After precipitation for 2 h at –20°C and centrifugation at 15 000 x *g* for 30 min, the DNA pellets were washed twice with 70% v/v ethanol and air-dried. DNA was redissolved in 20 µl dH_2O and used directly for amplification using the polymerase chain reaction (PCR).

An initial test of efficiency of amplification and the number of detected loci, was performed on four random isolates with ten RAPD primers (Table 2). Reproducibility of amplification was tested by varying PCR conditions and concentrations, and using genomic DNA isolated on separate days. Potential primers were used in PCR with genomic DNA from each isolate in the study. Each RAPD PCR reaction contained 5 µl 10x PCR Reaction Buffer, 2·5 mM $MgCl_2$, 200 µM of each dNTP, 2 µM RAPD primer, 5U of SuperTherm *Taq* polymerase (Southern Cross Biotechnology, South

Africa), and 1 µl (~5 ng) of DNA solution. The reaction volume was adjusted to 50 µl and overlaid with mineral oil to prevent evaporation. Thermal cycling was performed on a Hybaid OmniGene (Hybaid, U.K.) thermal cycler, and constituted an initial denaturation at 94°C for 2 min 30 s, followed by 35 cycles of primer annealing at 35°C for 45 s, DNA synthesis at 72°C for 1 min 30 s and denaturation at 94°C for 30 s. The protocol was terminated after a final extension at 72°C for 7 min. Two microliters of each reaction product were electrophoresed on a 1% w/v analytical grade agarose (Promega Corporation, Madison, USA) gel at 3 V/cm for 4 h. The gel was stained with ethidium bromide (Sambrook *et al.*, 1989) and fingerprints visualised and photographed using an ultraviolet transilluminator. Fingerprints were scored as multilocus haplotypes (genotypes) to evaluate genotypic diversity. Individual bands were scored for presence or absence in order to facilitate neighbour-joining analysis for comparison with VCG data.

Statistical analyses

Both data sets obtained from VCG and RAPD analyses, were subjected to distance analysis with a heuristic search protocol, using the PAUP* 4 computer programme. Unrooted neighbour-joining trees were generated with the Goloboff fit criterion (*K*) set to "2".

For the purpose of comparison with results from previous studies, the Shannon index (*SI*) of phenotypic diversity (Anagnostakis *et al.*, 1986; Cortesi *et al.*, 1996) was calculated for the set of VCG data, using the formula

$$SI = -\sum_{i=1}^{k} p_i \ln p_i$$

where p_i is the frequency of the i^{th} phenotype, k is the number of phenotypes (VCGs) in the population, and ln p_i is the natural logarithm of p_i. The Shannon index approximates zero when little variation is present, and the sample number as diversity increases. The index was normalised with

$$H_s = \frac{SI}{\ln(N)}$$

where H_s is the normalised Shannon index (SI), and N is the sample size (Sheldon, 1969). Genotypic diversity using RAPD multilocus haplotypes and VCG data was calculated with

$$G = \frac{1}{\sum_{x=0}^{N}\left[f_x\left(\frac{x}{N}\right)^2\right]}$$

where N is the sample size and f_x is the number of genotypes observed x times in the population (Stoddart & Taylor, 1988). Values were corrected for sample size by calculating the maximum percentage of genotypic diversity, $\hat{G}$ (McDonald *et al.*, 1994):

$$\hat{G} = (G/N)$$

The significance of differences between the percentages of maximum diversity obtained for VCG data and RAPD data was calculated using a t-test (Chen *et al.*, 1994):

$$t = \frac{\left|\frac{\hat{G}_1}{N_1} - \frac{\hat{G}_2}{N_2}\right|}{\sqrt{\frac{Var(\hat{G}_1)}{N_1^2} + \frac{Var(\hat{G}_2)}{N_2^2}}}$$

where

$$Var(\hat{G}) = \frac{4}{N} G^2 \left[G^2 \sum_{i=0}^{K} (p_i)^3 - 1 \right]$$

G is the genotypic diversity obtained for each data set;

K is the number of genotypes in the sample;

p_i is the frequency of the i^{th} genotype in the sample;

$\hat{G}$ is the maximum likelihood estimator for G in this equation (Stoddart & Taylor, 1988).

The t-test was calculated at a significance level of 99% (P = 0·01). The degrees of freedom were $N_1 + N_2 - 2$.

RESULTS

Fungal cultures

Fifty-nine single ascospore isolates of *C. cubensis* were obtained from the two sampled plantations in Colombia. Twenty-nine isolates originated from plantation A (CRY1061 - CRY 1084, CRY1451 - CRY1455), while 30 originated from plantation B (CRY1090, CRY1101, CRY1113, CRY1125, CRY1137, CRY1144, CRY1164, CRY1173, CRY1185, CRY1197, CRY1209, CRY1221, CRY1231, CRY1258, CRY1266, CRY1278, CRY1290, CRY1303, CRY1309, CRY1326, CRY1349, CRY1364, CRY1377, CRY1386, CRY1400, CRY1404, CRY1422, CRY1424, CRY1436, CRY1457).

Vegetative compatibility groups

Barrage formation between isolates of different VCGs could be scored easily and consistently between replicates (Figure 1). Weak barrage interactions were detectable by viewing the Petri dishes on a light box, which revealed faint green lines and green discoloration of peripheral mycelium between incompatible isolates. Scoring of VCG interactions revealed isolates that grouped consistently within a VCG, but did not form confluent mycelium with any of the isolates in other VCGs (Table 1).

RAPD DNA fingerprinting

Preliminary tests to identify potential RAPD primers resulted in the identification of three primers that yielded more than 10 bands. These primers, namely UBC181, UBC285 and UBC566 (Table 2), yielded bands that could be clearly distinguished from each other. The reproducibility of these profiles was good, with the exception of bands smaller than 600 bp that did not amplify consistently. These bands were excluded from further analyses. A total of 47 characters were scored (Figure 2) when profiles from the three primers were combined: 21 characters from primer UBC181, 14 from UBC285, and 12 from UBC566. Band sizes ranged from 600 bp to >2600 bp (Figure 3).

Statistical analyses

Neighbour-joining distance analyses of scored VCG and RAPD data resulted in two unrooted trees, one each for VCG data (Figure 4) and RAPD data (Figure 5).

Grouping of isolates within VCGs was supported by the tree resulting from VCG data. Only the grouping of isolates in VCG 1 was supported across both trees (Table 1, Figures 4 & 5).

The Shannon diversity index for the 31 isolates within 15 discrete VCGs was 3·8, and H_s was 1·1. The maximum percentage of genotypic diversity for these isolates was calculated at 37%. When RAPD data were analysed, the statistic was 100%, since every isolate had a distinct genotypic fingerprint. Using a t-test, a significant difference ($P = 0.01$) in the percentage of maximum genotypic diversity was found for isolates in discrete VCGs, when RAPD data were compared with VCG data.

DISCUSSION

Based on the results of this study, it is clear that the Colombian population of *C. cubensis* consists of numerous VCGs. Fifteen VCGs, represented by 31 isolates, were identified. In comparison, Van Heerden (1999) found that a South African population of the fungus was represented by only 23 VCGs out of 100 isolates tested. For the Colombian population tested in this study, the normalised Shannon index (H_s) was calculated at 1·1. The highest value reported for this statistic in the South African population, was 0·990 (Van Heerden, 1999). The Colombian population of *C. cubensis* is, therefore, more diverse than that of the fungus in South Africa.

The genotypic diversity of the Colombian *C. cubensis* agrees with that found for other South American populations (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998). In the present study, however, RAPD data were used for the first time to estimate the

genotypic diversity of a *C. cubensis* population from South America. Values for the maximum percentage of genotypic diversity ($\hat{G}$) based on RAPD and VCGs were significantly (P = 0.01) different, with RAPD data showing a much higher level of diversity (100%) than VCG data (37%). This result can be explained when the genetic bases of the two techniques are considered. The RAPD technique is based on the random amplification of DNA with arbitrary primers (Williams *et al.*, 1990), and represents a sub-sample of the entire genome. In contrast, vegetative compatibility is based on testing interactions between a limited number of genes at the *vic* loci (Leslie, 1993). Therefore, it is conceivable that a population might possess a limited number of *vic* genes, but that each VCG consists of numerous genotypes. Although VCG tests in this study show considerable diversity in the Colombian *C. cubensis* population, RAPD data suggests that this is probably an underestimation of diversity.

The transmission of double-stranded RNA viruses between isolates of *C. cubensis* (Van Heerden, 1999), as with *C. parasitica* (Cortesi *et al.*, 1996; Heiniger & Rigling, 1994), depends on their VCG associations, and not their relatedness. When two isolates represent the same VCG, the hypovirulence agent can be readily transferred between them. However, Van Heerden (1999) showed that the *C. parasitica* hypovirus can be transferred between *C. cubensis* isolates in different VCGs. This suggests that hypovirulence as biocontrol agent may succeed in populations of the fungus that consist of only a few VCGs. Control of Cryphonectria canker in Colombia would consequently not be viable, due to the high level of VCG divergence observed in this population.

Future investigations into the biology of populations of *C. cubensis* will benefit by the development of characterised molecular markers. These could aid in investigations of panmixia, *i.e.* random mating in the absence of selective pressure, and gametic disequilibrium (Burdon & Roelfs, 1985a; Hartl & Clark, 1989; McDonald *et al.*, 1994; Milgroom, 1996) of sexually reproducing populations, such as those found in South America and Asia (Florence *et al.*, 1986; Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998). A closer study of population subdivision (Milgroom & Lipari, 1995) and other external factors such as migration (Milgroom, 1995), would also be possible.

REFERENCES

Alfenas, A. C., Jeng, R. & Hubbes, M. (1984). Isozyme and protein patterns of isolates of *Cryphonectria cubensis* differing in virulence. *Canadian Journal of Botany* **62**, 1756-1762.

Anagnostakis, S. L. (1982). Genetic analysis of *Endothia parasitica*: linkage data for four single genes and three vegetative compatibility types. *Genetics* **102**, 25-28.

Anagnostakis, S. L., Hau, B. & Kranz, J. (1986). Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. *Plant Disease* **70**, 536-538.

Anagnostakis, S. L. & Kranz, J. (1987). Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. *Phytopathology* **77**, 751-754.

Bayman, P. & Cotty, P. J. (1991). Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. *Canadian Journal of Botany* **69**, 1707-1711.

Bégueret, J., Turq, B. & Clavé, C. (1994). Vegetative incompatibility in filamentous fungi: *het* genes begin to talk. *Trends In Genetics* **10**, 441-445.

Beisson-Schecroun (1962). Incompatibilité cellulaire et interactions nucléo-cytoplasmiques dans les phénomènes de barrage chez le *Podospora anserina*. *Annales de Genetique* **4**, 3-50.

Bernet, J. (1965). Mode d'action des gènes de barrage et relation entre l'incompatibilité cellulaire et l'incompatibilité sexuelle chez *Podospora anserina*. *Annales des Sciences Naturelles, Botanique, Paris* **12**, 611-768.

Brasier, C. M. (1987). Recent genetic changes in the *Ophiostoma ulmi* population: the threat to the future of the elm. In *Populations of Plant Pathogens: their Dynamics and Genetics*, pp. 213-226. Edited by M. S. Wolfe & C. E. Caten. Oxford: Blackwell Scientific Publications.

Burdon, J. J. & Roelfs, A. P. (1985a). The effect of sexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* **75**, 1068-1073.

Burdon, J. J. & Roelfs, A. P. (1985b). Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. *Phytopathology* **75**, 907-913.

Chen, R.-S., Boeger, J. M. & McDonald, B. A. (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**, 209-218.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990). Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**, 43-49.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1992). Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. *European Journal of Forest Pathology* **22**, 312-315.

Correll, J. C., Gordon, T. R. & McCain, A. H. (1992). Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Phytopathology* **82**, 415-420.

Cortesi, P., Milgroom, M. G. & Bisiach, M. (1996). Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. *Mycological Research* **100**, 1087-1093.

Davison, E. M. & Coates, D. J. (1991). Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157-160.

Florence, E. J. M., Sharma, J. K. & Mohanan, C. (1986). A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384-386.

Francis, D. M. & St Clair, D. A. (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. *Current Genetics* **24**, 100-106.

Goodwin, S. B., Drenth, A. & Fry, W. E. (1992). Cloning and genetic analysis of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Current Genetics* **22**, 107-115.

Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995). A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied And Environmental Microbiology* **61**, 3943-9.

Hartl, D. L. & Clark, A. G. (1989). *Principles of population genetics*, Second Edition. Sunderland, Massachusetts, USA: Sinauer Associates, Inc.

Heiniger, U. & Rigling, D. (1994). Biological control of chestnut blight in Europe. *Annual Review of Phytopathology* **32**, 581-599.

Hodges, C. S. (1980). The taxonomy of *Diaporthe cubensis*. *Mycologia* **72**, 542-548.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology* **31**, 127-150.

McDonald, B. A., Miles, J., Nelson, L. R. & Pettway, R. E. (1994). Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology* **84**, 250-255.

Melchinger, A. E. (1990). Use of molecular markers in breeding for oligogenic disease resistance. *Plant Breeding* **104**, 1-19.

Milgroom, M. G. (1995). Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany* **73(Suppl. 1)**, S311-S319.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

Milgroom, M. G. & Lipari, S. E. (1995). Population differentiation in the chestnut blight fungus, *Cryphonectria parasitica*, in Eastern North America. *Phytopathology* **85**, 155-160.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y. (1993). Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **70**, 385-392.

Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992). DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **131**, 297-306.

Milgroom, M. G., Lipari, S. E. & Wang, K. (1992). Comparison of genetic diversity in the chestnut blight fungus, *Cryphonectria* (*Endothia*) *parasitica*, from China and the U.S. *Mycological Research* **96**, 1114-1120.

Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4325.

Myburg, H. (1997). *Cryphonectria cubensis*, a molecular taxonomic and population study. Department of Microbiology and Biochemistry. Bloemfontein: University of the Orange Free State. *M.Sc. Thesis*.

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

Newhouse, J. R. & MacDonald, W. L. (1991). The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Canadian Journal of Botany* **69**, 602-614.

Powell, W. A. (1995). Vegetative incompatibility and mycelial death of *Cryphonectria parasitica* detected with a pH indicator. *Mycologia* **87**, 738-741.

Rizwana, R. & Powell, W. A. (1995). Ultraviolet light-induced heterokaryon formation and parasexuality in *Cryphonectria parasitica. Experimental Mycology* **19**, 48-60.

Sambrook, J., Fritch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A laboratory manual*, Second Edition. USA: Cold Spring Harbor Laboratory Press.

Scibilia, K. L. & Shain, L. (1989). Protection of American chestnut with hypovirulent conidia of *Cryphonectria* (*Endothia*) *parasitica. Plant Disease* **73**, 840-843.

Sheldon, A. L. (1969). Equitability indices: dependence on the species count. *Ecology* **50**, 466-467.

Smith, J. M. (1968). Evolution in sexual and asexual populations. *The American Naturalist* **102**, 469-473.

Stoddart, J. A. & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics* **118**, 705-711.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis*.

Van Heerden, S. W., Wingfield, M. J., Coutinho, T. & Van Zyl, L. M. (1997). Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv. (Abstract).

Van Zyl, L. M. (1995). Some factors affecting the susceptibility of *Eucalyptus* spp. to *Cryphonectria cubensis*. Department of Microbiology and Biochemistry. Bloemfontein: University of the Orange Free State. *M.Sc. Thesis*.

Van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998). Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**, 41-47.

Whisson, S. C., Drenth, A., Maclean, D. J. & Irwin, J. A. (1994). Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Current Genetics* **27**, 77-82.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Wingfield, M. J., Van Zyl, L. M., Van Heerden, S., Myburg, H. & Wingfield, B. D. (1997). Virulence and the genetic composition of the *Cryphonectria cubensis*

Bruner population in South Africa. In *Physiology and genetics of tree-phytophage interactions*, pp. 163-172. Edited by F. Lieutier, W. J. Mattson & M. R. Wagner. Gujan, France: INRA Editions.

Table 1. VCG associations of isolates of *C. cubensis* from Colombia.

VCG Number	Isolate Number (CRY Number)
1	CRY 1084
	CRY 1173
	CRY 1290
2	CRY 1074
3	CRY 1400
4	CRY 1066
	CRY 1075
	CRY 1221
	CRY 1231
5	CRY 1068
	CRY 1070
	CRY 1078
6	CRY 1077
	CRY 1113
	CRY 1144
	CRY 1266
7	CRY 1422
	CRY 1209
	CRY 1364
8	CRY 1137
	CRY 1424
9	CRY 1079
	CRY 1404
	CRY 1197
10	CRY 1071
	CRY 1377
11	CRY 1451
12	CRY 1065
13	CRY 1457
14	CRY 1454
15	CRY 1061

Table 2. RAPD primers screened and used to fingerprint isolates of *C. cubensis* representing a random population sample from Colombia.

Primer Name	Sequence (5' – 3')
UBC181[1]	ATG ACG ACG G
UBC285[1]	GGG CGC CTA G
UBC350	TGA CGC GCT C
UBC372	CCC ACT GAC G
UBC566[1]	CCA CAT GCG A
OPD02	GAG GAT CCC T
OPE12	TTA TCG CCC C
OPF01	ACG GAT CCT G
OPF02	GAG GAT CCC T
OPF12	ACG GTA CCA G

[1] Primers yielding more than 10 bands for identification of individual genotypes and scoring of loci.

UBC: University of British Columbia, USA

OPD, OPE and OPF: Operon Technologies, Inc.

Figure 1. Binary scores of VCG interactions between Colombian isolates of *C. cubensis* when paired in all possible combinations.

	CRY1061	CRY1062	CRY1451	CRY1065	CRY1066	CRY1068	CRY1070	CRY1071	CRY1074	CRY1075	CRY1077	CRY1078	CRY1079	CRY1454	CRY1221	CRY1400	CRY1404	CRY1422	CRY1113	CRY1137	CRY1144	CRY1173	CRY1197	CRY1209	CRY1231	CRY1424	CRY1266	CRY1290	CRY1457	CRY1364	CRY1377
CRY1061	0																														
CRY1062	1	0																													
CRY1451	1	1	0																												
CRY1065	1	1	1	0																											
CRY1066	1	1	1	1	0																										
CRY1068	1	1	1	1	1	0																									
CRY1070	1	1	1	1	1	0	0																								
CRY1071	1	1	1	1	1	1	1	0																							
CRY1074	1	1	1	1	1	1	1	1	0																						
CRY1075	1	1	1	1	0	1	1	1	1	0																					
CRY1077	1	1	1	1	1	1	1	1	1	1	0																				
CRY1078	1	1	1	1	1	0	0	1	1	1	1	0																			
CRY1079	1	1	1	1	1	1	1	1	1	1	1	1	0																		
CRY1454	1	1	1	1	1	1	1	1	1	1	1	1	1	0																	
CRY1221	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0																
CRY1400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0															
CRY1404	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0														
CRY1422	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0													
CRY1113	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0												
CRY1137	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0											
CRY1144	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0										
CRY1173	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0									
CRY1197	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0								
CRY1209	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0							
CRY1231	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0						
CRY1424	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0					
CRY1266	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0				
CRY1290	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0			
CRY1457	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0		
CRY1364	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	
CRY1377	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0

Figure 2. Raw data from the scoring of RAPD profiles of *C. cubensis* isolates. A total of 48 characters were scored when profiles from three RAPD primers were combined.

```
[                    1         10        20        30        40        ]

CRY1061              00000010010101010111000000001000001100110010111
CRY1062              10000010101010101011001110100010010nnnnnnnnnnnnn
CRY1451              00101010111010101111101101010010110100000010110
CRY1065              00101010111010101111101101110010110010011001011O
CRY1066              00101010110001011011011011100011101000000000100
CRY1068              01101010110101011011011011101011101nnnnnnnnnnnnn
CRY1070              00000000011100000011000000101100101100110010110
CRY1071              01100010110101011011011011101001101100110010110
CRY1074              00100000110101011111000011101001101000000010111
CRY1075              01100010110101010011011011101101101nnnnnnnnnnnnn
CRY1077              01101010110111011111011011101100101100110010110
CRY1078              01000001010100000011111010100101110nnnnnnnnnnnnn
CRY1079              01100010110101011010000000100010010000010010110
CRY1454              00000010110101011111001010101100101nnnnnnnnnnnnn
CRY1221              01000000010101000011000001101000101nnnnnnnnnnnnn
CRY1400              01100010110101011011011011101100101100101010111
CRY1404              01100110110101011010000001101000101000000000110
CRY1422              01100010110101010011011010101001101100000010110
CRY1113              01100011110101010111011010101000101100110010111
CRY1137              00100010110101011011001001101001101000000000110
CRY1144              01000011011101010011Onnnnnnnnnnnnnnn000110010110
CRY1173              10000111010110100100000110100010000001000101001
CRY1197              01000000010101000010011011101000101010111010111
CRY1209              00101010110101011010Onnnnnnnnnnnnnn000010010110
CRY1231              01101010110111011011001011101001101000010010111
CRY1266              00100110110101011010001010101000101100110010111
CRY1290              10000110100111100010000110000110001nnnnnnnnnnnn
CRY1457              00000000000000010111000001100000111000000010110
CRY1364              00100110110101011011001001101000101000010010110
CRY1377              01100010110101011011011010100100111010101O110
```

Figure 3. RAPD fingerprints of a representative sample of isolates from the Colombian population of *C. cubensis* (inverted colours). The fingerprints were obtained by random amplification with primer UBC181 during PCR, followed by electrophoresis on a 1% w/v analytical grade agarose gel at 3 V/cm for 4 h. Molecular weight sizes are indicated at the left of the gel. Lanes 1, 14 and 27 contain a 100 bp DNA ladder, while the rest of the lanes contain fingerprints from 24 isolates of *C. cubensis*.

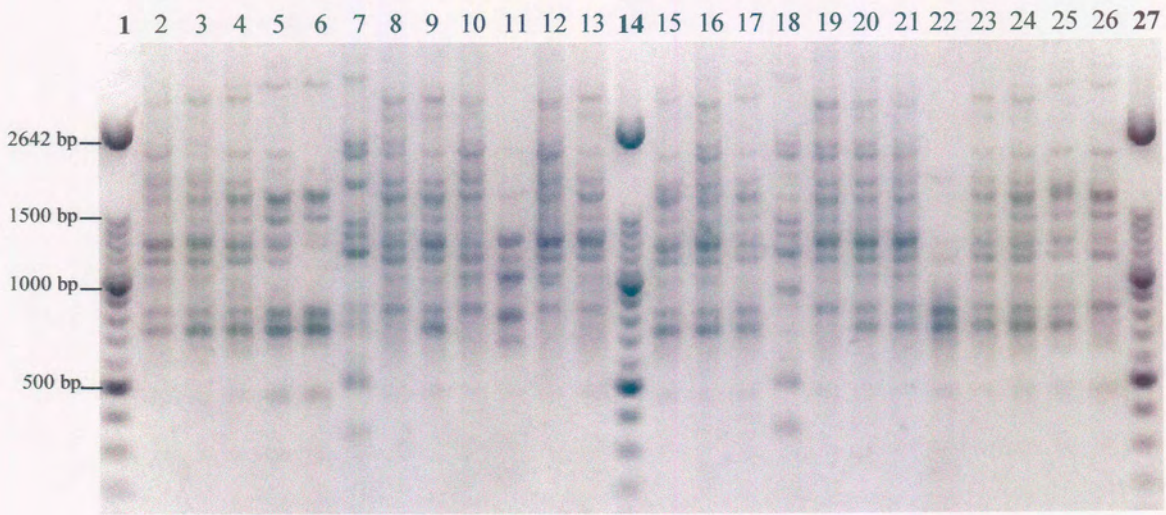


Figure 4. Neighbour-joining tree of VCG data. Isolates that group into VCGs also group together in this tree (shaded areas with black border, VCG numbers indicated).

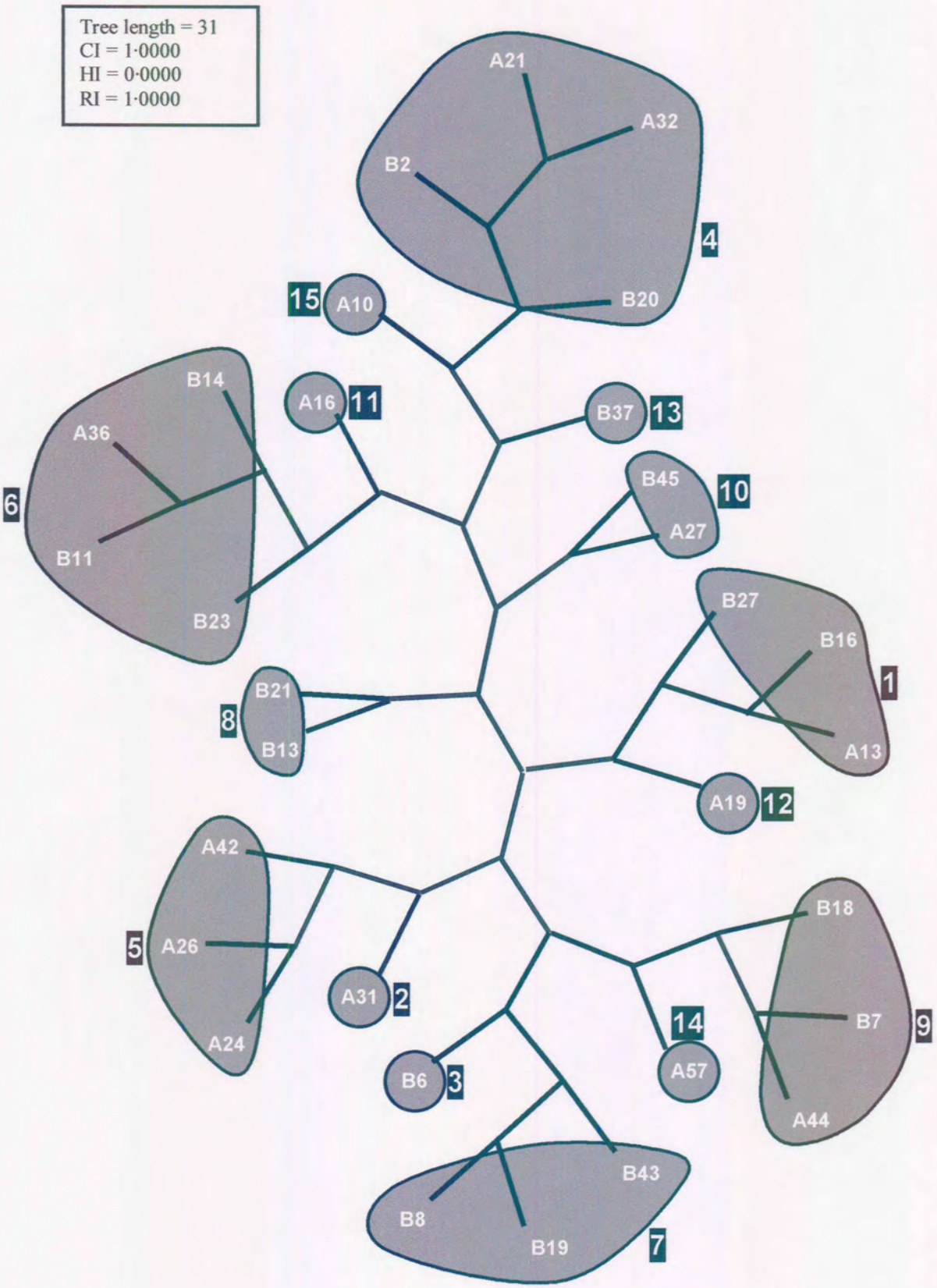
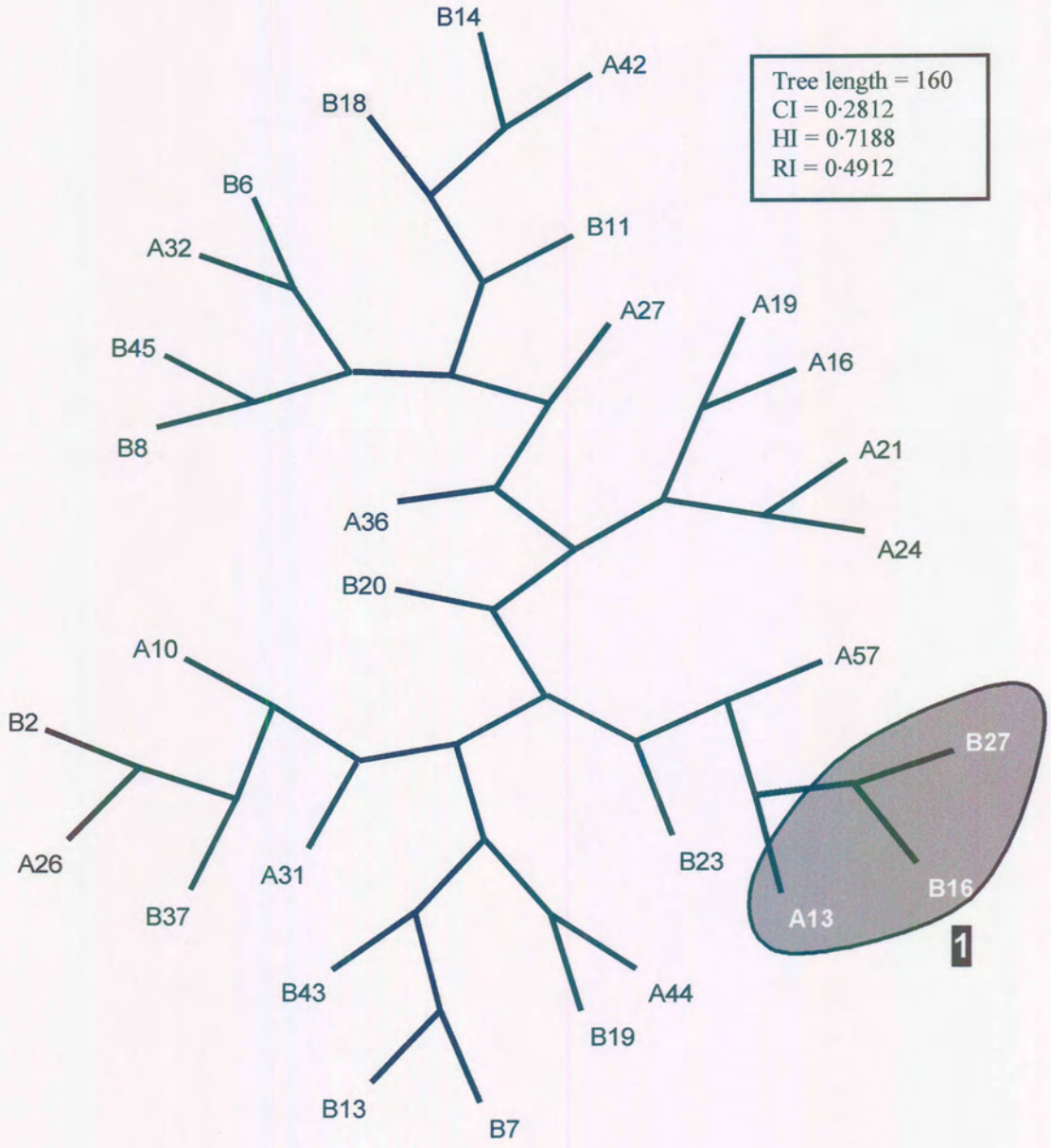
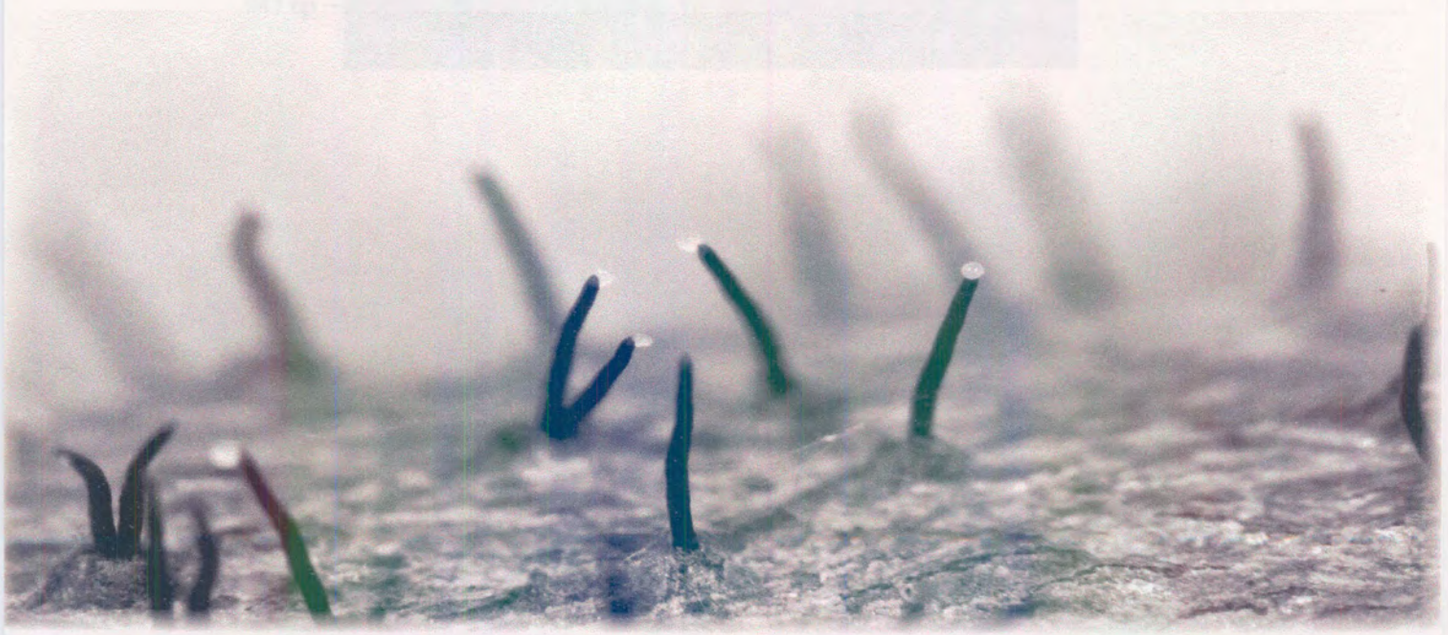

Tree length = 31
CI = 1·0000
HI = 0·0000
RI = 1·0000
A21
A32
B2
4
B20
15
A10
B14
A16
11
B37
13
A36
6
B45
10
A27
B11
B23
B27
B16
1
B21
8
B13
A13
A19
12
A42
B18
5
A26
A31
2
B7
9
14
A24
B6
3
A57
A44
B43
B8
B19
7

Figure 5. Neighbour-joining tree generated using RAPD data. Isolates from VCG 1 grouped together in the same fashion as with VCG data, but none of the other VCGs that are represented by more than one isolate, were supported.



CHAPTER V

SEQUENCE CHARACTERISED POLYMORPHIC MARKERS DIFFERENTIATE BETWEEN *CRYPHONECTRIA CUBENSIS* ISOLATES FROM SOUTH AFRICA AND SOUTH EAST ASIA



SEQUENCE CHARACTERISED POLYMORPHIC MARKERS DISCRIMINATE BETWEEN ISOLATES OF *CRYPHONECTRIA CUBENSIS* FROM SOUTH AFRICA, SOUTH AMERICA AND SOUTH EAST ASIA

ABSTRACT

Cryphonectria cubensis is an important stem canker pathogen of *Eucalyptus* in most tropical and sub-tropical parts of the world where this tree is grown. In the past, isolates of *C. cubensis* from South Africa, South America and South East Asia have been studied at the DNA level. Phylogenetic analyses using sequence data from the internal transcribed spacer (ITS) region, have revealed that isolates from different continents are closely related, and that South African *C. cubensis* isolates group in a South American clade. It was thus hypothesised that *C. cubensis* has been introduced into South Africa from South America. The aim of this study was, therefore, to develop polymerase chain reaction (PCR) -based DNA markers that will enable the separation of South African *C. cubensis* isolates from isolates originating in other countries. Genomic DNA from a South African isolate of *C. cubensis* was used in inter-short sequence repeat (ISSR)-PCR, and all the amplification products were cloned. Twenty-one clones were sequenced and used to design specific primers. After PCR amplification was verified, one primer of each pair was fluorescently labelled to yield marked PCR amplification products. These fragments were electrophoresed using an automated sequencer and analysed for polymorphisms between *C. cubensis* isolates from different countries. A high percentage of the original clones yielded markers that are polymorphic for isolates of *C. cubensis* originating in South Africa and elsewhere. Three of the eight markers amplified only in South African isolates, and not in isolates from other countries. These results show that *C. cubensis* from South Africa is very different to the fungus in South America and South East Asia. The taxonomy of the two groups of fungi thus deserve further investigation.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges occurs in tropical and sub-tropical regions of the world (Florence *et al.*, 1986; Hodges *et al.*, 1979; Wingfield *et al.*, 1989) where it causes an important stem canker disease of *Eucalyptus* trees (Conradie *et al.*, 1990; Conradie *et al.*, 1992; Florence, *et al.*, 1986). The fungus has been isolated from stems of both *Eucalyptus* and *Eugenia* trees in South America (Hodges *et al.* 1986) and South East Asia (Sharma *et al.*, 1985), but is unusual in that it also occurs on the roots of *Eucalyptus* in Australia (Davison & Coates, 1991; Myburg *et al.*, 1999). In South Africa, *C. cubensis* is pathogenic on *Eucalyptus* trees and causes substantial damage in plantations (Wingfield *et al.*, 1989).

Isozyme comparisons have shown that *C. cubensis* is the same as the clove canker pathogen known as *Endothia eugeniae* (Nutman & Roberts) Reid and Booth (Alfenas *et al.*, 1984). This was confirmed in a recent phylogenetic study based on internal transcribed spacer (ITS) sequence data (Myburg *et al.*, 1999). These authors also showed that *C. cubensis* from South Africa is more closely related to the fungus from South America than that from South East Asia.

In South Africa, *C. cubensis* is primarily associated with basal cankers on young trees. These usually kill trees in the first year of growth and cankers on the stems of older trees are never seen (Conradie *et al.*, 1990; Conradie, *et al.* 1992; Wingfield *et al.*, 1989). This is very different to symptoms in South America and South East Asia, where basal cankers and cankers higher up on stems of established trees are common (Florence *et al.*, 1986; Hodges *et al.*, 1979; Sharma *et al.*, 1985). Cankers on trees in

South America and South East Asia tend to be covered with perithecia of *C. cubensis*. In contrast, perithecia are virtually never seen on cankers in South Africa (M.J. Wingfield, *unpublished*). These differences in symptoms associated with *C. cubensis* suggest that the fungus in South Africa might be different to that in other parts of the world.

DNA fingerprinting techniques such as randomly amplified microsatellites (RAMS) (Hantula *et al.*, 1996), amplified fragment length polymorphisms (AFLPs) (Hill *et al.*, 1996), and randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) have been used extensively for identification of individuals in populations. However, in order to discriminate between isolates from diverse origins, it is necessary to analyse the occurrence of polymorphic alleles at specific loci. Several molecular techniques, including isozyme typing (Alfenas *et al.*, 1984), restriction fragment length polymorphisms (RFLPs) (Milgroom & Lipari, 1993; Milgroom *et al.*, 1993) and microsatellite analyses (Hughes & Queller, 1993), can be employed to study polymorphic alleles.

DNA-based markers have been used in many organisms (Puterka *et al.*, 1993; Selander *et al.*, 1985; Struss & Plieske, 1998), including fungi (Francis & St Clair, 1993; Gobbi *et al.*, 1990; Groppe *et al.*, 1995). The use of polymorphic RFLP markers, for instance, has provided insight into sexual outcrossing frequencies (Milgroom *et al.*, 1993), and population diversity (Milgroom *et al.*, 1992; Milgroom & Lipari, 1995) of the chestnut blight pathogen, *Cryphonectria parasitica*. Other fungi have also been studied using DNA markers, to demonstrate homothallic outcrossing (Francis & St Clair, 1993; Whisson *et al.*, 1994).

The discovery of short sequence repeats (SSRs, microsatellites) in higher eukaryotes (Lagerkrantz *et al.*, 1993) has prompted a major thrust in DNA marker technology. SSRs also occur in fungi, and have been described from *Podospora anserina* (Osiewacz *et al.*, 1996), *Microbotryum violaceum* (Delmotte *et al.*, 1999) and several of the endophytic *Epichloë* spp. (Groppe *et al.*, 1995; Moon *et al.*, 1999). These sequences can be exploited as highly polymorphic DNA markers in the identification of individuals (Baleiras Couto *et al.*, 1996), kinship determinations (Queller *et al.*, 1993), and studies of species that were previously difficult to analyse at the population level (Delmotte *et al.*, 1999).

The aim of this study was to develop and characterise microsatellite-like DNA markers that are polymorphic for isolates of *C. cubensis* from South Africa, South America and South East Asia. Markers were isolated from inter-short sequence repeat (ISSR; randomly amplified microsatellites, RAMS) (Hantula *et al.*, 1996) PCR amplification products from a South African isolate of *C. cubensis*, and tested on isolates of *C. cubensis* from these geographical regions. Isolates of *C. cubensis* originating from clove (*Eugenia carophyllus*), as well as two *C. parasitica* isolates, were included in the analyses for comparative purposes.

MATERIALS AND METHODS

Fungal cultures

Ten *C. cubensis* isolates from different geographical origins and plant hosts were used in this study (Table 1). Two isolates of a closely related species, *C. parasitica* isolated from American chestnut trees in the USA, were also included for comparison. All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

DNA isolation and ISSR-PCR

Genomic DNA was isolated from all cultures using the protocol described by Murray & Thompson (1980). DNA pellets were suspended in 20 µl sterile water, treated with 10 µg RNAse H at 37°C for 2 h to remove residual RNA, and then stored at –20°C until further use.

Inter-short sequence repeat (ISSR) PCR was performed on a HybAid OmniGene thermocycler (HybAid, UK). Three ISSR primers, namely BDB$(ACA)_5$, DHB$(CGA)_5$, and HV$(GT)_8$, were used to test for the number of polymorphic bands amplified, between two South African isolates of *C. cubensis*. Each PCR reaction contained 1 µl (~25 ng) of isolated genomic DNA from one of the two South African isolates, 5 µl 10x PCR buffer, 25 mM $MgCl_2$, 200 µM of each dNTP, 5 U SuperTherm *Taq* Polymerase (Southern Cross Biotechnology, South Africa), and 2 µM of the ISSR primer. Reaction volumes were adjusted to 50 µl with sterile water and overlaid with

mineral oil to prevent evaporation. Cycling conditions included denaturation at 96°C for 1 min, annealing at 47°C (BDB{ACA}$_5$), 59°C (DHB{CGA}$_5$)or 56°C (HV{GT}$_8$) for 1 min, and extension at 72°C for 1 min, repeated for 30 cycles. A final extension of the PCR product was done at 72°C for 6 min. Ten µl of each PCR reaction mix was electrophoresed on a 1% w/v agarose gel containing 0·5 µg/ml ethidium bromide. Amplification products were visualised under ultraviolet light.

Cloning and screening

PCR amplification products from isolate CRY620 (South Africa), generated with primer ACA, were purified with a NucleoSpin Extract PCR purification kit (Macherey-Nagel, GmbH). These fragments were cloned into the pGEM®-T vector using the pGEM®-T Easy Vector System (Promega, USA) and transformed into *Escherichia coli* JM109 super-competent cells as described by the manufacturer. Clones were screened to assess their plasmid insert size using alkaline lysis and restriction enzyme analysis (Sambrook *et al.*, 1989). Only plasmids containing inserts in the size range of 200-1000 bp (base pairs) were chosen for further analysis.

Sequencing and primer design

Candidate plasmids containing potential microsatellite sequences were sequenced with universal primers T7 and SP6, using a Dye Primer Cycle Sequencing Kit (Perkin Elmer, CA) and ABI Prism™ 377 automatic sequencer. Resulting electropherograms were analysed using the Sequence Navigator computer programme, and primers designed around regions showing microsatellite-like organisation. The distance

between the primers was in the range of 150 bp to 450 bp. Primers were usually 18 bases or more in length, with G+C content approximating 50% and, therefore, annealing temperatures (T_a) between 48°C and 55°C (Table 3).

Primer testing and marker identification

Primer pairs designed from the microsatellite sequences were used in PCR with genomic DNA from all isolates included in the study. PCR was performed on a HybAid OmniGene (HybAid, UK) thermocycler, with each PCR reaction containing 1 µl (~25 ng) genomic DNA, 400 nM of each primer, 250 µM of each dNTP, 25 mM $MgCl_2$, and 2·5 U SuperTherm *Taq* Polymerase (Southern Cross Biotechnology, South Africa). PCR reaction volumes were adjusted to 25 µl with the addition of sterile water and overlaid with mineral oil to prevent evaporation. The PCR programme involved an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C (30 s), primer annealing at 45°C (30 s), and extension at 72°C (1 min). The reaction was completed with a final extension at 72°C for 7 min.

Five µl of each PCR amplification product was electrophoresed on a 6% denaturing polyacrylamide sequencing gel (Sambrook *et al.*, 1989), which was subsequently silver stained (Bassam & Cactano-Anollés, 1993) to visualise amplification of a DNA fragment in the expected size range. One primer from each primer pair that yielded a single PCR product of the expected size was labelled with one of the phosphoramidite fluorescent dyes, 6-FAM or TET. The fluorescently labelled primers were used in subsequent PCR reactions. These fluorescent products were electrophoresed on a GeneScan® gel to analyse differences in product size between all of the isolates used in

the study. Each PCR product was assigned a locus name, with product sizes differentiating alleles at each locus. GENESCAN-500™ TAMRA (Perkin Elmer, CA) was used as a size standard in all lanes.

RESULTS

ISSR-PCR

Inter-short sequence repeat PCR with three primers, BDB(ACA)$_5$, DHB(CGA)$_5$ and HV(GT)$_8$, yielded amplification products with genomic DNA from both South African *C. cubensis* isolates. A low level of polymorphism in banding patterns between these isolates was observed when primers DHB(CGA)$_5$ and HV(GT)$_8$ were used. Primer BDB(ACA)$_5$ produced profiles that differed extensively, with *ca.* 10% of the bands being shared between the isolates.

Cloning and screening

Transformation of *E. coli* JM109 cells with plasmids containing the BDB(ACA)$_5$ PCR reaction amplification products from isolate CRY620, yielded 56 *E. coli* clones with plasmids that contained inserts. Screening of these clones by plasmid isolation and restriction enzyme digestion revealed that 21 of the clones contained inserts in the size range between 200 bp and 1000 bp (Figure 1). Therefore, a third of the transformants contained plasmids with inserts that could be entirely sequenced using the universal primers, SP6 and T7.

Sequencing and primer design

Twenty of the 21 plasmids containing small inserts were sequenced entirely. One plasmid contained an insert with a putative GC stretch, making it difficult to obtain sequence information for the entire insert. All of the inserts showed a highly repetitive sequence organisation, but no perfect dinucleotide repeats of longer than four were observed (Figure 2). Eleven PCR primer pairs were designed from 11 of the clones.

Primer testing and marker identification

Of the eleven primer pairs designed from plasmid insert sequences, only eight gave reproducible PCR products (Table 3). Primer pairs CCMS7/CCMS8, CCMS11/CCMS12, and CCMS17/CCMS18 did not produce PCR amplification products. PCR with genomic DNA from two Brazilian, two South African, a Thai and an Australian *C. cubensis* isolate resulted in amplification of polymorphic PCR products, when analysed on GeneScan® gels (Table 4). None of the labelled primer pairs produced amplification products with genomic DNA from the two *C. parasitica* isolates, and *C. cubensis* isolates CRY 164 (Brazil), CRY 544 (Indonesia), CRY 1082 (Colombia), and CRY 1085 (Colombia).

Primers designed for loci *CcPM-B* (*Cryphonectria cubensis* Polymorphic Marker B), *CcPM-E* and *CcPM-G* yielded PCR products only with South African *C. cubensis* isolates. Alleles at loci *CcPM-A* and *CcPM-J* were polymorphic between isolates from different origins. Primers for locus *CcPM-J* amplified four alleles, namely $CcPM\text{-}J_{216}$, $CcPM\text{-}J_{230}$, $CcPM\text{-}J_{233}$ and $CcPM\text{-}J_{241}$.

DISCUSSION

Data emerging from this study have shown that South African isolates of *C. cubensis* are alike, but that they are very different from those from South America and South East Asia. The level of difference is great and suggests that the fungi, although closely related (Myburg *et al.*, 1999), might have undergone speciation. Because *C. cubensis* is known to have appeared in South Africa relatively recently (Wingfield *et al.*, 1989), it seems unlikely that this could have been linked to the geographic barriers between South America and South Africa. A more reasonable explanation would be that *C. cubensis* in South Africa represents a species very similar to the fungus in South America, but one that has yet to be discovered in the latter area.

Cryphonectria cubensis in South Africa is known to possess some very distinct differences to the fungus in South America and South East Asia. In South Africa, the sexual state is virtually never seen on cankers on *Eucalyptus* trees, whereas these are the predominant structures in South America and South East Asia (Hodges *et al.*, 1979). In the latter areas the fungus is associated with swelling of the bases of trees and cankers are generally found high on the stems. In contrast, the fungus in South Africa is most common at the bases of stems and generally kills trees in the first year of growth (Van Heerden, 1999; Wingfield *et al.*, 1989). The very distinct differences between South African and other isolates of *C. cubensis*, that have emerged in this study, are consistent with the notion that these groups of the fungus are distinct.

A South African isolate of *C. cubensis* was used in this study to develop polymorphic markers. These markers are valuable tools for the analysis of South African *C. cubensis* at the population level. However, due to the fact that this fungus has emerged as being different from that in South East Asia and South America, the markers will probably not be useful for population analyses of the fungus from those areas. For this purpose, additional polymorphic markers, developed from the South American and South East Asian isolates, will be needed.

The markers developed in this study have microsatellite-like sequence organisation, but no more than four repeats of a dinucleotide motif were observed. The length polymorphisms of amplified alleles at the tested loci, however, do not reflect changes in repeat numbers of these microsatellite-like regions. Polymorphisms can rather be attributed to a combination of this type of stepwise mutation (Goldstein *et al.*, 1995; Lehmann *et al.*, 1996), and random insertions and deletions of single bases over the length of the alleles. The microsatellite-like polymorphic markers developed during this study will be useful for studies of *C. cubensis*, even though the exact mutational mechanisms are unknown.

In order to facilitate a detailed study of the frequencies of specific alleles in populations of *C. cubensis* from geographically diverse origins, additional isolates from different areas should be included. These data will also allow for the elucidation of gene flow between populations (Hartl & Clark, 1989), and an investigation into random mating mechanisms (Milgroom *et al.*, 1992; Milgroom, 1996) within populations. A larger number of characterised loci will be of value when such studies are undertaken. In addition, the mechanisms of mutation, which are responsible for

polymorphisms between alleles, must be investigated in order to carry out statistical analyses of data.

REFERENCES

Alfenas, A. C., Jeng, R. & Hubbes, M. (1984). Isozyme and protein patterns of isolates of *Cryphonectria cubensis* differing in virulence. *Canadian Journal of Botany* **62**, 1756-1762.

Baleiras Couto, M. M., Hartog, B. J., Huis in't Veld, J. H. J., Hofstra, H. & Van der Vossen, J. M. B. M. (1996). Identification of spoilage yeasts in a food-production chain by microsatellite polymerase chain reaction fingerprinting. *Food Microbiology* **13**, 59-67.

Bassam, B. J. & Cactano-Anollés, G. (1993). Silver staining of DNA in polyacrylamide gels. *Applied Biochemistry and Biotechnology* **42**, 181-188.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990). Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**, 43-49.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1992). Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. *European Journal of Forest Pathology* **22**, 312-315.

Davison, E. M. & Coates, D. J. (1991). Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157-160.

Delmotte, F., Bucheli, E. & Shykoff, J. A. (1999). Host and parasite population structure in a natural plant-pathogen system. *Heredity* **82**, 300-308.

Florence, E. J. M., Sharma, J. K. & Mohanan, C. (1986). A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384-386.

Francis, D. M. & St Clair, D. A. (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. *Current Genetics* **24**, 100-106.

Gobbi, E., Wang, Y., Martin, R. M., Powell, W. A. & Van Alfen, N. K. (1990). Mitochondrial DNA of *Cryphonectria parasitica*: lack of migration between vegetatively compatible strains. *Molecular Plant-Microbe Interactions* **3**, 66-71.

Goldstein, D. B., Linares, A. R., Cavalli-Sforza, L. L. & Feldman, M. W. (1995). An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139**, 463-471.

Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995). A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied And Environmental Microbiology* **61**, 3943-9.

Hantula, M., Dusabenyagasani, M. & Hamelin, R. C. (1996). Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* **26**, 159-166.

Hartl, D. L. & Clark, A. G. (1989). *Principles of population genetics*, Second Edition. Sunderland, Massachusetts, USA: Sinauer Associates, Inc.

Hill, M., Witsenboer, H., Zabeau, M., Vos, P., Kesseli, R. & Michelmore, R. (1996). PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics* **93**, 1202-1210.

Hodges, C. S., Alfenas, A. C. & Ferreira, F. A. (1986). The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**, 343-350.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

Hughes, C. R. & Queller, D. C. (1993). Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. *Molecular Ecology* **2**, 131-137.

Lagerkrantz, U., Ellegren, H. & Andersson, L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**, 1111-1115.

Lehmann, T., Hawley, W. A. & Collins, F. H. (1996). An evaluation of evolutionary contraints on microsatellite loci using null alleles. *Genetics* **144**, 1155-1163.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

Milgroom, M. G. & Lipari, S. E. (1993). Maternal inheritance and diversity of mitochondrial DNA in the chestnut blight fungus, *Cryphonectria parasitica*. *Phytopathology* **83**, 563-567.

Milgroom, M. G. & Lipari, S. E. (1995). Population differentiation in the chestnut blight fungus, *Cryphonectria parasitica*, in Eastern North America. *Phytopathology* **85**, 155-160.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y. (1993). Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **70**, 385-392.

Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992). DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **131**, 297-306.

Milgroom, M. G., Lipari, S. E. & Wang, K. (1992). Comparison of genetic diversity in the chestnut blight fungus, *Cryphonectria* (*Endothia*) *parasitica*, from China and the U.S. *Mycological Research* **96**, 1114-1120.

Moon, C. D., Tapper, B. A. & Scott, B. (1999). Identification of *Epichloë* endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**, 1268-1279.

Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4325.

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

Osiewacz, H. D., Hamann, A. & Werner, A. (1996). Genome analysis of filamentous fungi: identification of a highly conserved simple repetitive sequence in different strains of *Podospora anserina*. *Microbiological Research* **151**, 1-8.

Puterka, G. J., Black, W. C., Steiner, W. M. & Burton, R. L. (1993). Genetic variation and phylogenetic relationships among worldwide collections of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), inferred from allozyme and RAPD-PCR markers. *Heredity* **70**, 604-618.

Queller, D. C., Strassman, J. E. & Hughes, C. R. (1993). Microsatellites and kinship. *TREE* **8**, 285-288.

Sambrook, J., Fritch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A laboratory manual*, Second Edition. USA: Cold Spring Harbor Laboratory Press.

Selander, R. K., McKinney, R. M., Whittam, T. S., Bibb, W. F., Brenner, D. J., Nolte, F. S. & Pattison, P. E. (1985). Genetic structure of populations of *Legionella pneumophila*. *Journal of Bacteriology* **163**, 1021-1037.

Sharma, J. K., Mohanan, C. & Florence, E. J. M. (1985). Occurrence of Cryphonectria canker disease of *Eucalyptus* in Kerala, India. *Annals of Applied Biology* **106**, 265-276.

Struss, D. & Plieske, J. (1998). The use of microsatellite markers for detection of genetic diversity in barley populations. *Theoretical and Applied Genetics* **97**, 308-315.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis*.

Whisson, S. C., Drenth, A., Maclean, D. J. & Irwin, J. A. (1994). Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. *Current Genetics* **27**, 77-82.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Table 1. Isolates used in this study.

Culture Collection Number	Species	Origin	Host
CRY 611	*Cryphonectria cubensis*	KwaZulu Natal, South Africa	*Eucalyptus grandis*
CRY 620	..	..	..
CRY 1085	..	Cali, Colombia	..
CRY 1082	..	..	..
CRY 500	..	Valença, Brazil	*Eugenia carophyllus*
CRY 544	..	Sumatera, Indonesia	..
CMW 2628	..	Australia	*Eucalyptus* spp.
CMW 3746	..	Thailand	*Eucalyptus deglupta*
CRY 164	..	Aracruz, Brazil	*Eucalyptus grandis*
CRY 302	..	Bahia Sul, Brazil	..
CMW 1652	*Cryphonectria parasitica*	USA	*Castanea dentata*
CMW 1654	..	..	..

All isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Insititute (FABI), University of Pretoria, Pretoria.

Table 2. PCR primers designed from sequenced inserts of plasmid clones. The inserts originated from an ISSR-PCR product obtained from isolate CRY 620.

Primer Name[a]	Sequence (5'-3')	Expected annealing and melting temperature[b]			Length (bases)	Expected Product Size (bp)[c]
		T_m (°C)	T_a (°C)	GC%		
CCMS1-A (FAM)	ttggaagcttaccatttcac	54·8	49·8	40·0	20	**439**
CCMS2-A	tatggaaatgacgcttcttg	54·8	49·8	40·0	20	
CCMS3-B (TET)	gttcataggctatcaaatcc	54·8	49·8	40·0	20	**312**
CCMS4-B	ctggtgtcattgcaaatc	51·8	46·8	44·4	18	
CCMS5-C (FAM)	ttgcgtatggaaatgacg	51·8	46·8	44·4	18	**223**
CCMS6-C	atggcgcttgtatagagca	54·2	49·2	47·4	19	
CCMS7-D	gtatctcagttcggtgtag	54·2	49·2	47·4	19	(173)
CCMS8-D	ggttctgctaatccttgtac	55·2	50·2	45·0	20	
CCMS9-E (TET)	gatctagcatctatcctgtc	55·2	50·2	45·0	20	**427**
CCMS10-E	ttcgcaggtgtagtcatgtc	55·6	50·6	50·0	20	
CCMS11-F	gtttgctcaggcatctattg	55·2	50·2	45·0	20	(285)
CCMS12-F	ccttatcaaggactgctatg	55·2	50·2	45·0	20	
CCMS13-G (FAM)	tgattcacgtctattgccac	55·2	50·2	45·0	20	**302**
CCMS14-G	gttaagttctcggtgaatcg	55·2	50·2	45·0	20	
CCMS15-H (FAM)	cattggagcacttcacattc	55·2	50·2	45·0	20	**306**
CCMS16-H	cagtatccggtttcctaatg	55·2	50·2	45·0	20	
CCMS17-I	caaagctttcgattcagatg	54·8	49·8	40·0	20	(441)
CCMS18-I	gttcatatcgattcgaaagg	54·8	49·8	40·0	20	
CCMS19-J (TET)	agcttagcactttgtcttgg	55·2	50·2	45·0	20	**229**
CCMS20-J	acgggatttcgatcagtttc	55·2	50·2	45·0	20	
CCMS21-K (TET)	cgctctctcatttgtgagtatc	58·6	53·6	45·5	22	**393**
CCMS22-K	gtaagggttcaagctttcacag	58·6	53·6	45·5	22	

[a] Alphabetic suffixes presented in uppercase indicate primer pairs.

[b] T_a and T_m represent the expected annealing and melting temperatures in °C, respectively.

[c] Expected product sizes of primer pairs that did not produce PCR products are indicated in brackets, while those that successfully produced PCR products are indicated in bold.

Table 3. Alleles observed for isolates of *C. cubensis* from different geographical origins. Allele sizes are indicated in base-pairs.

Isolate Number	Isolate Identity	Origin	Host	Locus											
				CcPM-A		*CcPM-B*	*CcPM-C*	*CcPM-E*	*CcPM-G*	*CcPM-H*	*CcPM-J*				*CcPM-K*
CMW 1652	*C. parasitica*	USA	*Castanea dentata*	-	-	-	-	-	-	-	-	-	-	-	-
CMW 1654	*C. parasitica*	USA	..	-	-	-	-	-	-	-	-	-	-	-	-
CRY 1082	*C. cubensis*	Colombia	*Eucalyptus grandis*	-	-	-	-	-	-	-	-	-	-	-	-
CRY 1085	..	Colombia	..	-	-	-	-	-	-	-	-	-	-	-	-
CRY 164	..	Brazil	..	-	-	-	-	-	-	-	-	-	-	-	-
CRY 544	..	Indonesia	*Eugenia carophyllus*	-	-	-	-	-	-	-	-	-	-	-	-
CRY 302	..	Brazil	*Eucalyptus grandis*	413	-	-	216	-	-	-	-	-	-	-	-
CRY 500	..	Brazil	*Eugenia carohyllus*	-	423	-	216	-	-	-	216	-	233	-	373
CMW 2628	..	Australia	*Eucalyptus* spp.	-	-	-	216	-	-	307	216	-	233	241	373
CMW 3746	..	Thailand	*Eucalyptus deglupta*	-	423	-	216	-	-	307	216	-	233	-	373
CRY 611	..	South Africa	*Eucalyptus grandis*	-	423	359	216	416	300	307	216	230	-	-	373
CRY 620	..	South Africa	..	-	423	359	216	416	300	307	216	230	-	241	373

Figure 1. Plasmid DNA digested with *Eco*RI to reveal insert sizes. Digested plasmid DNA was electrophoresed on a 1% w/v agar gel at 6 V/cm for 1 h. Clone numbers are indicated at the top of the gel. Fragment sizes are indicated on the left.

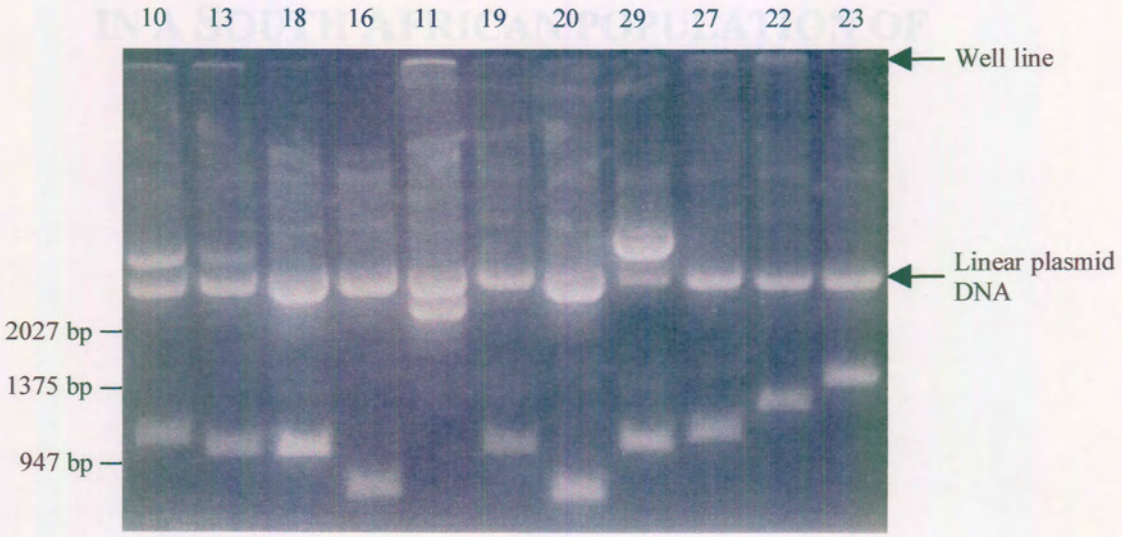


Figure 2. DNA sequence of loci *CcPM-A* (A), *-B* (B), *-C* (C), *-E* (D), *-G* (E), *-H* (F), *-J* (G), and *-K* (H). Primer binding sites are indicated in inverted colours.

A [redacted]caaccaagttcatatcgattcgaaaggcttgacaccttccgctggaaggcatagttaagcgttgatgactttcctctctctgtcgcaatgccagtagtacggcaaccacctgcaaaaactcccaa
tcatatcgccgatgtgggctgtgccatccaccagcccaagagtaccactgtcggtgatcacaaacggcacgaccgcacatggcgctgtatagagcattggttatacccatttagcccattcttcattacacgggcttgccttcca
tctagcaggaccaagcgcttccatagcaaattagctgtgtgtgggatcaagaactggttgcggcttcggatggacctctctttccggatgcgagcggcgactagagtgattcggaaagcgccctcgaga[redacted]
[redacted]

B [redacted]tgaccgagcatgttgcgcggtttcgaaaatggaagagcctaaggcgtatgcatcatcaacggttgaggtagtcttctggcagagtagtcttctgtcatnaggcaactagctcgtgatcgaccggt
gacctagtggtcgtgtaggctctctatcgtatgagagtcagtnctcgacgtcgtgggtgttattcctgtgaagctgacctactctcacagcatcctatcatttatcttcctgatctgctacattgcttccgaaatcgcgcgacat
caaa[redacted]

C [redacted]cttcttgtctcgagggcgctttccgaatcactctagtcgccgctcggcatccggaaagagaggtccatccgaagccgcaacagttcttgatcccacacacagctaattttgctatggaagcgcttgg
tcctgctagatggaaggcaagcccgtgtaatgaagaatggggctaaatggtataaccaa[redacted]

D [redacted]actagacatgatcgacgaaatcatatcggacccacaaaaattgcggctggcactctgggtttatctgccgcacaatcatcagccgccatcatggcttatagtaagtcgcgattaggcagtactg
ctgcgaactatttggaattggcagatgctgatgaaaggtgaacagcacatggcattagaaacatatactacttcatgatcccatgctgctgtatttccttcttctcacacttttcttcgttagaggccacagtttgaagagagaa
gatgatgccgaagctacaggaagaaggcagacgatgggcagcgagtcgtgaaggggttattgaatcgcgaaaaggaatccaggcagaaaagtctttccgatcgacacgccggtcggc[redacted]

E [redacted]ttttgcgacaaggactatatgactcgcggcgcagcacgttttcactatcagcaggcaagaagacaaaagctctctagcattactcttttttctggccgactcactgaccccccttcagagccaca
ccaagaagcagcttattgcttgccctggctgtaatgtccctaatgccaaggtctcggcttttgtcaagcacatcgagcttggcttgctgtcccaggatcaccccgcagatggccgccgacgcttgccagacaaacaa[redacted]
[redacted]

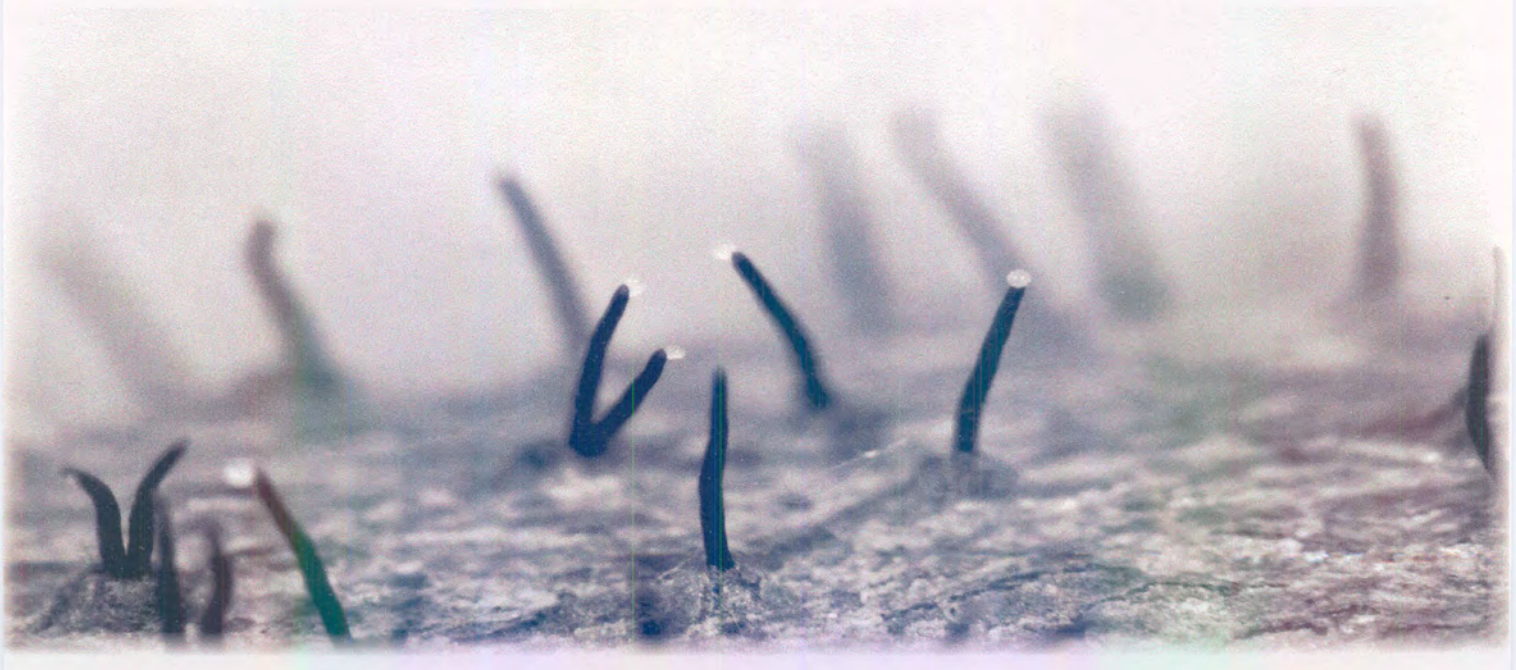
F [redacted]tcgggttgatatgttccccgcctgagggatatagcgataccgcagtcgctattatcaggcagatactcgtccagtggtggtgctctggtgtctttagaggaatgcactgctgaaaggccttgga
tggaaggtgtatcaaatagtctcacgccataacagatactttcttcacgccgtgcaagtgaggactacggtacagtgtatttataatcattattgagcttggtagcttactgcacgctacggccagctgttcagaattgt[redacted]
[redacted]

G [redacted]ctatataaagccattgtgaccatagtcaggtaaagggctcatctttcagcaaatggcggggcccgctgggcacgtagggatatataaaatcctgggaagctagacgatctcgagcactgtagtct
cctccttctcaatcccgagactacctagatctcatcaatatcctacaacacagataaaaacaat[redacted]

H [redacted]cctctcgacagaaaactcttgtgtaacattcttttcatccaagctgacgtgggaggatggaatcaagtctgccaaatctgttgggttcataggctatcaaatcctgaccgagcatgttgccg
ccggttcgaaaatgggaagaacctaagggcgtatgccatcatcaacggcttggaaggtaagtcttctgggcaacaagtagtctttctggtcattcaggccaacttagctcgctgtatcgggcacccgggcttgcaccttcagtgg
ggtcgtgggtagggctctctatcgtaatgaggtaggtccgaggntctcggaccgttcgtggggngntttaatttc[redacted]

CHAPTER VI

GENOTYPIC DIVERSITY AND GAMETIC EQUILIBRIUM IN A SOUTH AFRICAN POPULATION OF *CRYPHONECTRIA CUBENSIS*



GENOTYPIC DIVERSITY AND GAMETIC EQUILIBRIUM IN A SOUTH AFRICAN POPULATION OF *CRYPHONECTRIA CUBENSIS*

ABSTRACT

Cryphonectria cubensis is a serious pathogen of plantation *Eucalyptus* in South Africa. The fungus causes cankers at the bases of stems, resulting in restriction of water and nutrient transport and, ultimately, death of trees. The diversity of the South African population of *C. cubensis* has previously been studied at the phenotypic level, using vegetative compatibility groups (VCGs). However, the recent development of characterised molecular markers for this pathogen has enabled a more detailed study of the population. The aim of this study was to determine the genetic diversity of *C. cubensis* in South Africa, and to test the hypothesis that the population is reproducing asexually and is, therefore, in gametic disequilibrium. These questions were addressed using DNA markers. Genomic DNA from 34 isolates of *C. cubensis* from South Africa, representing 23 VCGs, was used in PCR with specific fluorescently labelled primers for eight polymorphic loci. Alleles for each locus were identified and used to construct multilocus haplotypes (genotypes). Gene and genotypic diversity were calculated using the frequencies of alleles and genotypes in the population. Gametic disequilibrium was determined by calculating the linkage of alleles at different polymorphic loci. Results indicated that both the gene diversity (H = 11·4%) and the maximum percentage of genotypic diversity ($\hat{G}$ = 11·5%) was very low. The gametic disequilibrium test, however, revealed that alleles at the loci approximate random association (D = 0·012). Therefore, the previous generation gave rise to the observed generation through genetic recombination. These findings contradict the fact that sexual structures of *C. cubensis* are rarely seen in South Africa. We believe that the detection of genetic recombination in this population might be due to several factors, including multiple introductions of the fungus into the country, sexual reproduction on an alternative host, or parasexual recombination.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is a pathogen that causes a serious canker disease on *Eucalyptus* trees in tropical and sub-tropical parts of the world (Conradie *et al.*, 1990; Florence *et al.*, 1986; Hodges *et al.*, 1979). Cankers at the bases of trees (Florence *et al.*, 1986; Wingfield *et al.*, 1989) are of concern to forestry companies that exploit *Eucalyptus* trees for pulp and timber production. Several tree-breeding programmes have consequently been initiated to breed and select trees resistant to Cryphonectria canker.

In South Africa, tree breeding relies on the identification of the most virulent isolates that can be used to screen for disease tolerance (Van Heerden, 1999). These isolates are identified from populations of which the diversity has been determined. It is important to gain knowledge of the population diversity of the pathogen, since very diverse populations can adapt to changes in host resistance more quickly than genetically uniform populations (Delmotte *et al.*, 1999; McDonald & McDermott, 1993).

The diversity of the South African population of *C. cubensis* has recently been assessed using vegetative compatibility groups (VCGs) (Van Heerden, 1999), a phenotypic character that is widely used to determine fungal population diversity (Anagnostakis & Kranz, 1987; Correll *et al.*, 1992; Leslie, 1993). Van Heerden (1999) indicated that several VCGs exist in the South African population, but the diversity was nonetheless very low. The study does, however, raise the question whether the

number of VCGs can be attributed to sexual reproduction (outcrossing), or to numerous introductions of the fungus into the country (Myburg *et al.*, 1999).

Sexual reproduction in *C. cubensis* is very rare in South Africa (Wingfield *et al.*, 1997). In South America and Asia, this fungus reproduces primarily through the production of sexual fruiting structures (perithecia) (Florence *et al.*, 1986; Hodges *et al.*, 1979). These populations display a high level of VCG diversity, with numerous VCGs being identified in small samples (Van Heerden *et al.*, 1997; Van Zyl *et al.*, 1998). It has further been shown that outcrossing occurs in a Colombian population of the fungus (Chapter III, *this thesis*), even though the fungus is homothallic (Hodges *et al.*, 1979). The study also revealed that the progeny of an outcrossing event could have the same VCG phenotype. Thus, outcrossing must be investigated at the DNA level, since VCGs are not sufficiently sensitive to detect the recombination of more than a small number of genes (Milgroom *et al.*, 1993; Milgroom, 1996).

Molecular markers have been employed successfully to study populations of several important plant pathogenic fungi (McDonald & McDermott, 1993). It has for example, been used to elucidate the mixed mating system of a population of *Cryphonectria parasitica* (Milgroom *et al.*, 1993), as well as to consider random mating and linkage of alleles (Milgroom *et al.*, 1992). The most widely used molecular markers are restriction fragment length polymorphism (RFLP) markers. These markers are hybridised to enzymatic digests of total genomic DNA from each individual under consideration (Bonfante *et al.*, 1997; Chen & McDonald, 1996; Chen *et al.*, 1994; Francis & St Clair, 1993), followed by the identification of alleles. Recently, PCR-

based detection of markers has become popular due to their ease of use (Geistlinger *et al.*, 1997; Harry *et al.*, 1998).

The aim of this study was to re-evaluate the population diversity of the South African population of *C. cubensis* at the molecular level. Gametic disequilibrium was also investigated. Both of these questions were addressed by using previously developed molecular markers that have been shown to be polymorphic between isolates of *C. cubensis* from different parts of the world.

MATERIALS AND METHODS

Fungal isolates

Isolates of *C. cubensis* from South Africa were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria. Thirty-four isolates, representing 23 vegetative compatibility groups (VCGs), were used (Table 1). The isolates originated from 10 plantations in the KwaZulu-Natal province, and represented a random sample of the *C. cubensis* population in that area.

DNA isolation

C. cubensis isolates were transferred to fresh malt extract agar (MEA, 20% w/v) in individual 9-cm diameter Petri dishes and incubated in the dark at 26°C for 3 days. Actively growing mycelium was removed from the edge of each culture and inoculated into 0·75 ml 20% w/v sterile malt extract broth in 1·5 ml microcentrifuge

tubes. The tubes were incubated in a horizontal position at 26°C in the dark for 3 days, after which the tubes were centrifuged (10 000 x *g* for 10 min) to collect fungal mycelium. Genomic DNA was isolated using the protocol described by Murray & Thompson (1980) and stored at –20°C until further use.

PCR amplification and electrophoresis

Polymorphic loci were amplified from genomic DNA using eight specific primer pairs (Table 2). Each PCR reaction consisted of 5 ng of isolated genomic DNA, 250 µM of each dNTP, 25 mM $MgCl_2$, 0·5 U SuperTherm *Taq* Polymerase (Southern Cross Biotechnology, South Africa), and 400 nM of each locus-specific primer. Reaction volumes were adjusted to 25 µl with the addition of distilled water, and overlaid with mineral oil to prevent evaporation during thermal cycling. PCR was performed on a Hybaid OmniGene (Hybaid, UK) thermocycler, and consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of primer annealing at 50°C (1 min), DNA synthesis at 72°C (1 min), and denaturation at 94°C (1 min). A final extension was carried out at 72°C for 5 min.

PCR amplification mixtures were diluted 1:30 by adding sterile water. A small sample of the diluted amplification reaction (0·5 µl) was mixed with 1·5 µl sequencing loading dye (Perkin Elmer, CA) and loaded onto automatic sequencing gels. GENESCAN-500 TAMRA was used as the internal size standard in all lanes. After electrophoresis, the gels were analysed for presence of PCR amplification products in the expected size range for each locus. Allele sizes were recorded for all isolates at all loci.

Statistical analyses

The frequencies of all detected alleles were calculated by dividing the number of times that the allele was observed, by the sample size. Using these figures, the gene diversity (Nei, 1973) was calculated with the formula

$$H = 1 - \sum_k x_k^2$$

where H is the gene diversity, and x_k is the frequency of the k^{th} allele. Gene diversity of a haploid population is defined as the probability of obtaining two different alleles at a locus when two individuals are randomly sampled from the population, and is equivalent to heterozygosity. The gene diversity approximates 1 for an extremely diverse population, and 0 for a uniform population. It is, therefore, a useful statistic to assess the diversity of a population.

For comparison to previously recorded VCG data, the Stoddard & Taylor (1988) index of genotypic diversity was calculated with

$$G = \frac{1}{\sum_{x=0}^{N}\left[f_x\left(\frac{x}{N}\right)^2\right]}$$

where G is the genotypic diversity, N is the sample size, and f_x is the number of genotypes observed x times in the population. The maximum percentage of genotypic diversity ($\hat{G}$) was calculated by dividing G with N.

Gametic (linkage) disequilibrium (D) was calculated for all combinations of polymorphic loci with

$$D_{ij} = P_{ij} - p_i p_j$$

where P_{ij} is the observed frequency of genotypes that have the A_iB_j combination of alleles at loci A and B, respectively, and p_ip_j are the frequencies of those alleles in the population (Hartl & Clark, 1989). Only alleles that occur at frequencies higher than 5%, were used for the calculation of *D*. The maximum value for *D* is reached when the population undergoes no recombination (linkage disequilibrium), but *D* approximates zero when recombination took place in the previous generation (linkage equilibrium). *D* is consequently an indirect indication of recombination in the previous generation, which results in the allele combinations and frequencies present in the observed generation.

RESULTS

PCR amplification and electrophoresis

PCR amplification products were obtained for all loci (Table 4) . One locus, namely *CcPM-B*, displayed a null allele with 10 of the 34 isolates. Only loci *CcPM-B*, *CcPM-C* and *CcPM-G* yielded polymorphic alleles in this population, with five, two, and four alleles, respectively. The other five loci produced monomorphic alleles in the expected size range.

Statistical analyses

At all three polymorphic loci, one allele occurred at a frequency much higher than any of the other alleles (Table 3). Using the frequency data of alleles, gene diversity (Nei, 1973) was calculated at 11·4%. When alleles were combined into multilocus

haplotypes, 11 different genotypes were observed. One genotype occurred 15 times, while seven other genotypes each occurred only once in the population. These data were subsequently used for the calculation of the Stoddard & Taylor index of maximum percentage of genotypic diversity ($\hat{G}$), which was 11·5%. Gametic disequilibrium (D), based on the tested polymorphic loci, was calculated at 0·012 over all alleles.

DISCUSSION

In this study, the population variation of *C. cubensis* in South Africa was re-evaluated by using specific molecular markers. The gene and genotypic diversity (H and $\hat{G}$) calculated for this population, were both very low. Even though these two values are almost identical, they differ from the genotypic diversity calculated by Van Heerden (1999). Based on VCG data, the maximum percentage of genotypic diversity for this population was 0·095% (Van Heerden, 1999), while the value calculated in the present study, using molecular data, was 11·5%.

The difference in estimated values for genotypic diversity between VCG and molecular data, can be attributed to the fact that vegetative incompatibility is a multilocus trait (Anagnostakis, 1982) that can be tested only at the phenotypic level. In contrast, specific DNA markers represent single-locus characters and are consequently co-dominant (Rafalski *et al.*, 1996). Also, the number of DNA markers can be increased to a level that satisfies statistical procedures (Delmotte *et al.*, 1999), while a fixed number of loci are tested with VCG analyses (Anagnostakis, 1982). Therefore,

VCG data tend to underestimate the true genotypic variation in a population, while DNA marker data result in a more accurate estimation.

This study represents the first attempt at calculating the gene diversity of a *C. cubensis* population. This statistic is an estimation of the heterozygosity within the population (Burdon & Roelfs, 1985; Coates, 1988; Nei, 1973), and can thus be used to quantify population variation. The value obtained for gene diversity (*H*) was 11·4%. This value is almost identical to the value for the maximum percentage of genotypic diversity, which was 11·5%. The two statistics are thus, equal measures of diversity in this population.

The maximum percentage of genotypic diversity (11·4%) in the South African *C. cubensis* population is low in comparison to populations of other randomly mating ascomycetes. For example, genotypic diversity reached 87% of its maximum possible value in a sexually reproducing population of *Mycosphaerella graminicola* (Chen & McDonald, 1996). However, the values for gene diversity and genotypic diversity of the South African *C. cubensis* population support the hypothesis that the population was established based on a small number of individuals. Milgroom *et al.* (1992) examined two populations of *C. parasitica* that satisfied this criterion, and found that the gene diversity in China was 22%, while it was 8% in the United States. The values obtained in this study therefore, present concrete evidence that *C. cubensis* was introduced into South Africa, and that it has undergone minimal recombination.

Recombination of alleles occurs in the South African population of *C. cubensis*. This finding is based on the random association of alleles at three polymorphic loci. Thus,

gametic equilibrium has been restored within one generation of random mating (Hartl & Clark, 1989). The measure for gametic disequilibrium (*D*) in the South African *C. cubensis* population resulted in a value of 0·012. Since *D* is an indirect measure of recombination, the relatively low value calculated in this study suggests that sexual recombination is present in the South African *C. cubensis* population, although it is perhaps not very common.

Several hypotheses can serve to explain the possibility of recombination in an apparently asexually reproducing population. These include multiple recent introductions, sexual reproduction on an alternative host, sexual reproduction on a non-visible part of the host, and recombination through parasexuality. All three of these possible sources of recombination could be applied to *C. cubensis* in South Africa. Parasexuality is a particularly attractive explanation, since it has been shown to occur in a close relative of *C. cubensis*, namely *C. parasitica* (Rizwana & Powell, 1995). During the parasexual cycle, two haploid nuclei from different individuals fuse to form a diploid zygote (Bal *et al.*, 1975; Garber & Beraha, 1965; Genovesi & MaGill, 1976; Papa, 1973; Papa, 1978). No perithecia are formed, but crossing over can occur within the zygote (Lhoas, 1967; Poulter *et al.*, 1981; Zeigler, 1998). Subsequent nuclear divisions, together with loss of chromosomes, results in a return to the haploid state (Bal *et al.*, 1975). Parasexual recombination has, however, not yet been shown to occur in *C. cubensis*.

This study has resulted in the elucidation of the population diversity and gametic equilibrium in the South African *C. cubensis* population. Although gametic equilibrium can not be explained fully, its existence is an indication that the population

is able to adapt to new conditions. Future investigations should focus on the further characterisation of the South African and other populations in order to determine the relative importance of asexual and sexual reproduction.

REFERENCES

Anagnostakis, S. L. (1982). Genetic analysis of *Endothia parasitica*: linkage data for four single genes and three vegetative compatibility types. *Genetics* **102**, 25-28.

Anagnostakis, S. L. & Kranz, J. (1987). Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. *Phytopathology* **77**, 751-754.

Bal, J., Bartnik, W., Goryluk, B. & Pieniazek, N. J. (1975). An easy way of obtaining *Aspergillus nidulans* haploids in the parasexual cycle using *N*-glycosyl polifungin. *Genetical Research* **25**, 249-252.

Bonfante, P., Lanfranco, L., Cometti, V. & Genre, A. (1997). Inter- and intraspecific variability in strains of the ectomycorrhizal fungus *Suillus* as revealed by molecular techniques. *Microbiological Research* **152**, 287-292.

Burdon, J. J. & Roelfs, A. P. (1985). The effect of sexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* **75**, 1068-1073.

Chen, R. S. & McDonald, B. A. (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics* **142**, 1119-1127.

Chen, R.-S., Boeger, J. M. & McDonald, B. A. (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**, 209-218.

Coates, D. J. (1988). Genetic diversity and population genetic structure in the rare Chittering grass wattle, *Acacia anomala* Court. *Australian Journal of Botany* **36**, 273-286.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990). Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**, 43-49.

Correll, J. C., Gordon, T. R. & McCain, A. H. (1992). Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Phytopathology* **82**, 415-420.

Delmotte, F., Bucheli, E. & Shykoff, J. A. (1999). Host and parasite population structure in a natural plant-pathogen system. *Heredity* **82**, 300-308.

Florence, E. J. M., Sharma, J. K. & Mohanan, C. (1986). A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384-386.

Francis, D. M. & St Clair, D. A. (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. *Current Genetics* **24**, 100-106.

Garber, E. D. & Beraha, L. (1965). Genetics of phytopathogenic fungi. XIV. The parasexual cycle in *Penicillium expansum*. *Genetics* **52**, 487-492.

Geistlinger, J., Weising, K., Kaiser, W. J. & Kahl, G. (1997). Allelic variation at a hypervariable compound microsatellite locus in the ascomycete *Ascochyta rabiei*. *Molecular and General Genetics* **256**, 298-305.

Genovesi, A. D. & MaGill, C. W. (1976). Heterokaryosis and parasexuality in *Pyricularia oryzae* Cavara. *Canadian Journal of Microbiology* **22**, 531-536.

Harry, D. E., Temesgen, B. & Neale, D. B. (1998). Codominant PCR-based markers for *Pinus taeda* developed from mapped cDNA clones. *Theoretical and Applied Genetics* **97**, 327-336.

Hartl, D. L. & Clark, A. G. (1989). *Principles of population genetics*, Second Edition. Sunderland, Massachusetts, USA: Sinauer Associates, Inc.

Hodges, C. S., Geary, T. F. & Cordell, C. E. (1979). The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter* **63**, 216-220.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology* **31**, 127-150.

Lhoas, P. (1967). Genetic analysis by means of the parasexual cycle in *Aspergillus niger*. *Genetical Research* **10**, 45-61.

McDonald, B. A. & McDermott, J. M. (1993). Population genetics of plant pathogenic fungi. *BioScience* **43**, 311-319.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y. (1993). Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **70**, 385-392.

Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992). DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **131**, 297-306.

Milgroom, M. G., Lipari, S. E. & Wang, K. (1992). Comparison of genetic diversity in the chestnut blight fungus, *Cryphonectria* (*Endothia*) *parasitica*, from China and the U.S. *Mycological Research* **96**, 1114-1120.

Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4325.

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999). Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243-250.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* **70**, 3321-3323.

Papa, K. E. (1973). The parasexual cycle in *Aspergillus flavus*. *Mycologia* **65**, 1201-1205.

Papa, K. E. (1978). The parasexual cycle in *Aspergillus parasiticus*. *Mycologia* **70**, 766-773.

Poulter, R., Jeffery, K., Hubbard, M. J., Shepherd, M. G. & Sullivan, P. A. (1981). Parasexual genetic analysis of *Candida albicans* by spheroplast fusion. *Journal of Bacteriology* **146**, 833-840.

Rafalski, J. A., Vogel, J. M., Morgante, M., Powell, W., Andre, C. & Tingey, S. V. (1996). Generating and using DNA markers in plants. In *Analysis of non-mammalian genomes - a practical guide*, pp. 75-134. Edited by B. Birren & E. Lai. New York: Academic Press.

Rizwana, R. & Powell, W. A. (1995). Ultraviolet light-induced heterokaryon formation and parasexuality in *Cryphonectria parasitica*. *Experimental Mycology* **19**, 48-60.

Stoddart, J. A. & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics* **118**, 705-711.

Van Heerden, S. W. (1999). Pathogenicity and variation amongst South African isolates of *Cryphonectria cubensis*. Faculty of Biological and Agricultural Sciences. Pretoria: University of Pretoria. *M.Sc. Thesis*.

Van Heerden, S. W., Wingfield, M. J., Coutinho, T. & Van Zyl, L. M. (1997). Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv. (Abstract).

Van Zyl, L. M., Wingfield, M. J., Alfenas, A. C. & Crous, P. W. (1998). Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management* **112**, 41-47.

Wingfield, M. J., Swart, W. J. & Abear, B. (1989). First record of Cryphonectria canker of *Eucalyptus* in South Africa. *Phytophylactica* **21**, 311-313.

Wingfield, M. J., Van Zyl, L. M., Van Heerden, S., Myburg, H. & Wingfield, B. D. (1997). Virulence and the genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In *Physiology and genetics of tree-phytophage interactions*, pp. 163-172. Edited by F. Lieutier, W. J. Mattson & M. R. Wagner. Gujan, France: INRA Editions.

Zeigler, R. S. (1998). Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* **36**, 249-275.

Table 1. Isolates of *C. cubensis* from South Africa used in this study.

VCG[a]	Isolate Number[b]	Plantation Name[c]
SA1	CRY 623	Nyalazi J8
..	CRY 627	Futululu
SA2	CRY 624	..
SA3	CRY 615	Safcol 27
SA4	CRY 614	..
SA5	CRY 620	..
SA6	CRY 625	Safcol 129
SA7	CRY 603	Mondi S
..	CRY 605	Palm Ridge
..	CRY 610	..
..	CRY 617	Mondi S
..	CRY 626	Nyalazi R11
SA8	CRY 616	Nyalazi
..	CRY 619	Safcol 56
..	CRY 621	..
..	CRY 782	..
SA9	CRY 604	Mondi O
..	CRY 609	Safcol 7
SA10	CRY 606	Mondi O
SA11	CRY 630	Futululu
SA12	CRY 607	Palm Ridge
SA13	CRY 608	..
SA14	CRY 613	Futululu
..	CRY 628	..
..	CRY 632	..
SA15	CRY 631	..
SA16	CRY 633	..
SA17	CRY 600	Palm Ridge
SA18	CRY 599	Nyalazi
SA19	CRY 598	..
SA20	CRY 601	..
SA21	CRY 602	Safcol 7
SA22	CRY 612	..
SA23	CRY 611	..

[a] VCG assignments are those of Van Heerden (1999).
[b] All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.
[c] All plantations are in the vicinity of Richards Bay in the KwaZulu-Natal province, South Africa.

Table 2. Loci amplified from *C. cubensis* with specific primer pairs during PCR.

Locus Name	Primer Name	Primer Sequence (5' – 3')
CcPM-A	CCMS1-A	ttggaagcttaccatttcac
	CCMS2-A	tatggaaatgacgcttcttg
CcPM-B	CCMS3-B	gttcataggctatcaaatcc
	CCMS4-B	ctggtgtcattgcaaatc
CcPM-C	CCMS5-C	ttgcgtatggaaatgacg
	CCMS6-C	atggcgcttgtatagagca
CcPM-E	CCMS9-C	gatctagcatctatcctgtc
	CCMS10-C	ttcgcaggtgtagtcatgtc
CcPM-G	CCMS13-G	tgattcacgtctattgccac
	CCMS14-G	gttaagttctcggtgaatcg
CcPM-H	CCMS15-H	tattggagcacttcacattc
	CCMS16-H	cagtatccggtttcctaatg
CcPM-J	CCMS19-J	agcttagcactttgtcttgg
	CCMS20-J	acgggatttcgatcagtttc
CcPM-K	CCMS21-K	cgctctctcatttgtgagtatc
	CCMS22-K	gtaagggttcaagctttcacag

Table 3. Alleles amplified for all eight loci with specific primer pairs. Data for all isolates used in the study are presented.

Isolate	VCG	*CcPM-A*	*CcPM-B*					*CcPM-C*		*CcPM-E*	*CcPM-G*				*CcPM-H*	*CcPM-J*		*CcPM-K*
		423-bp	307-bp	359-bp	366-bp	372-bp	Null	209-bp	216-bp	416-bp	288-bp	292-bp	300-bp	307-bp	307-bp	216-bp	230-bp	373-bp
CRY627	SA1	1					1		1	1			1		1	1	1	1
CRY623	..	1		1					1	1			1		1	1	1	1
CRY624	SA2	1		1					1	1		1			1	1	1	1
CRY615	SA3	1		1					1	1			1		1	1	1	1
CRY614	SA4	1	1						1	1			1		1	1	1	1
CRY620	SA5	1		1					1	1			1		1	1	1	1
CRY625	SA6	1					1		1	1			1		1	1	1	1
CRY603	SA7	1		1					1	1			1		1	1	1	1
CRY605	..	1					1		1	1			1		1	1	1	1
CRY610	..	1		1					1	1			1		1	1	1	1
CRY617	..	1		1					1	1			1		1	1	1	1
CRY626	..	1					1		1	1		1			1	1	1	1
CRY616	SA8	1					1		1	1			1		1	1	1	1
CRY619	..	1					1		1	1			1		1	1	1	1
CRY621	..	1		1				1		1			1		1	1	1	1
Safcol562.2	..	1					1		1	1	1				1	1	1	1
CRY604	SA9	1		1					1	1			1		1	1	1	1
CRY609	..	1		1					1	1	1				1	1	1	1
CRY606	SA10	1		1					1	1			1		1	1	1	1
CRY630	SA11	1		1					1	1			1		1	1	1	1
CRY607	SA12	1		1					1	1				1	1	1	1	1
CRY608	SA13	1				1			1	1	1				1	1	1	1
CRY613	SA14	1					1		1	1			1		1	1	1	1
CRY628	..	1		1					1	1			1		1	1	1	1
CRY632	..	1			1				1	1			1		1	1	1	1
CRY631	SA15	1					1	1		1			1		1	1	1	1
CRY633	SA16	1		1					1	1			1		1	1	1	1
CRY600	SA17	1		1					1	1			1		1	1	1	1
CRY599	SA18	1		1					1	1			1		1	1	1	1
CRY598	SA19	1		1					1	1			1		1	1	1	1
CRY601	SA20	1					1		1	1			1		1	1	1	1
CRY602	SA21	1		1					1	1			1		1	1	1	1
CRY612	SA22	1		1					1	1	1				1	1	1	1
CRY611	SA23	1		1					1	1	1				1	1	1	1

Table 4. Allele frequencies, sample variance, and statistics associated with the calculation of Nei's (1973) index of genotypic diversity.

Locus	Allele (bp)	Allele Frequency (*f*)	Precision of *f* (s^2)[a]	Sample Variance (*s*)[b]	*H* (Nei, 1973)[c]
CcPM-A	423	1·000	0·029	0·171	0·000
CcPM-B	307	0·029	0·001	0·029	
	359	0·618	0·007	0·083	
	366	0·029	0·001	0·029	
	372	0·029	0·001	0·029	
	null	0·294	0·006	0·078	0·529
CcPM-C	209	0·059	0·002	0·040	
	216	0·941	0·002	0·040	0·111
CcPM-E	416	1·000	0·029	0·171	0·000
CcPM-G	288	0·147	0·004	0·061	
	292	0·059	0·002	0·040	
	300	0·765	0·005	0·073	
	307	0·029	0·001	0·029	0·389
CcPM-H	307	1·000	0·029	0·171	0·000
CcPM-J[d]	216	1·000	0·029	0·171	0·000
CcPM-J[d]	230	1·000	0·029	0·171	0·000
CcPM-K	373	1·000	0·029	0·171	0·000
					***H* = 0·114**

[a] Precision of allele frequency estimates were calculated with $s^2 = (p \times q)/N$ where p is the frequency of the allele, q is the combined frequencies of all the other alleles, and N is the sample size. This statistic is an indication of the effect of sample size on allele frequency.

[b] Sample variance (s) is the square root of s^2, and is an indication of the confidence limit (standard deviation) of the estimate of allele frequency ($P = 0{\cdot}05$).

[c] Nei's genotypic diversity was calculated with the formula described in the text.

[d] Two alleles were detected for locus *CcPM-J* in all isolates tested. The primers for this locus, therefore, amplify two monomorphic loci in the South African population of *C. cubensis* when used in PCR with genomic DNA, and are treated as such during statistical analyses.

SUMMARY

Cryphonectria canker of *Eucalyptus*, caused by *Cryphonectria cubensis*, is considered to be one of the most important fungal diseases affecting Forestry in South Africa. This disease also occurs in other tropical and sub-tropical regions of the world, including South America, Australia and South East Asia. Due to the commercial importance of *C. cubensis*, several recent studies in South Africa have focused on the elucidation of population diversity and phylogenetic relationships of the fungus from diverse geographic origins. These studies have resulted in more effective tree breeding programmes, and the identification of the possible origin for *C. cubensis* in South Africa.

In this thesis, various aspects of the biology of *C. cubensis* in South Africa and Colombia are addressed. These studies have focused on the elucidation of the diversity of populations of the fungus occurring in Colombia and South Africa, as well as determination of the phylogenetic relationships of *C. cubensis* from Colombia. The sexual reproductive system of the fungus from Colombia has also been investigated.

In an investigation into the phylogenetic relationships of *C. cubensis* isolates from Colombia (Chapter II), it was found that these isolates are most closely related to other South American isolates. This finding suggests that properties displayed by populations in other South American countries can be extrapolated to the Colombian population, and *vice versa*. The Colombian isolates were distinct from *C. cubensis* found in South East Asia, implying a more distant relatedness between these isolates.

A study of homothallism and the possibility of sexual outcrossing in Colombian *C. cubensis* isolates (Chapter III), revealed that the fungus is homothallic. This was shown by allowing single ascospore isolates to reproduce sexually on *Eucalyptus* twigs, followed by genetic analysis of progeny using DNA fingerprinting. The DNA fingerprinting profiles of these progeny were identical, indicating that no outcrossing had occurred. The sexual event was, therefore, due to self-fertilisation. In contrast, when progeny from naturally occurring perithecia were analysed genetically using vegetative compatibility groups (VCGs) and DNA fingerprinting, results suggested that oucrossing had occurred, but only to a limited extent. *C. cubensis* in Colombia is therefore homothallic, but can outcross. Presumably, the same is true for other populations of the fungus in South America.

The genotypic diversity of the Colombian population of *C. cubensis* was investigated using VCGs and RAPDs. Results of this study indicated that the genotypic diversity of this population was similar to diversities previously found for other South American populations of *C. cubensis*. However, the phenotypic (VCG) and genetic (RAPD) data for the Colombian population were significantly different. The estimation of genotypic diversity based on RAPDs was significantly higher than the same figure for VCG data. The reason for the difference in obtained values is attributed to the low level of sensitivity of VCGs to detect genetic differences between isolates.

In Chapter V, a novel technique for obtaining polymorphic, microsatellite-like DNA markers from fungi is described. The technique is based on the identification and characterisation of polymorphic DNA fragments originating from a random amplification of microsatellite sequences using the polymerase chain reaction (PCR).

Sequence data from these fragments were used to construct specific primers to amplify polymorphic loci from genomic DNA. The technique has a high success rate in comparison to traditional techniques for the isolation of polymorphic markers. It was also shown that markers obtained with the new technique can be used to differentiate *C. cubensis* isolates originating in South Africa from those originating in other countries.

The last chapter (VI) of this thesis represents the first intensive molecular population diversity study of *C. cubensis* in South Africa. Using polymorphic markers from an earlier study (Chapter V), it was possible to assess the molecular variation of the South African population, and to compare this with phenotypic data obtained previously. It was found that molecular and phenotypic data yield different estimations of population diversity. An estimation of the gametic disequilibrium of the South African *C. cubensis* population revealed that the tested alleles were randomly associated. Such a situation is expected for populations that preferentially reproduce sexually, and consequently outcross. These figures are, however, only an indirect indication that outcrossing occurs in the South African population. Future studies on the South African *C. cubensis* population would need to be based on a larger number of markers, and should also include a greater number of isolates of the fungus.

Knowledge gained through the studies presented in this thesis will hopefully aid to develop more effective control strategies against Cryphonectria canker of eucalypts. However, new questions about the biology of *C. cubensis* have emerged that need urgent attention. Data from this thesis might, therefore, prove important to future studies of the fungus.

OPSOMMING

Cryphonectria cubensis, die oorsaak van Cryphonectria kanker van *Eucalyptus*, word geag as een van die belangrikste fungus-patogene in die Suid-Afrikaanse bosbou-industrie. Die siekte kom ook voor in ander tropiese en sub-tropiese gebiede, insluitende Suid-Amerka, Australië en Suid-Oos Asië. As gevolg van die kommersiële belang van *C. cubensis* is verskeie studies uitgevoer wat gefokus het op bepaling van populasie-diversiteit en die filogenetiese verwantskappe van die fungus afkomstig uit diverse geografiese gebiede. Hierdie studies het gelei tot die implementering van meer effektiewe boomtelingsprogramme, asook bepaling van 'n moontlike oorsprong vir *C. cubensis* in Suid-Afrika.

In hierdie tesis word verskeie aspekte met betrekking tot die biologie van *C. cubensis* in Colombië aangespreek. Hierdie studies het gefokus op die bepaling van populasie-diversiteit van die fungus in beide Colombië en Suid-Afrika, asook bepaling van die filogenetiese verwantskappe van die fungus in Colombië. Die geslagtelike voortplantingsisteem van *C. cubensis* in Colombië is ook ondersoek.

Gedurende 'n ondersoek na die filogenetiese verwantskappe van *C. cubensis* isolate uit Colombië (Hoofstuk II), is vasgestel dat hierdie isolate die naaste verwant is aan ander Suid-Amerikaanse isolate. Die bevinding dui daarop dat die eienskappe van populasies in ander Suid-Amerikaanse lande moontlik deurgetrek kan word na die Colombiaanse populasie, en andersom. Die Colombiaanse isolate was onderskeibaar van *C. cubensis* afkomstig van Suid-Oos Asië, en dit impliseer 'n meer verwyderde verwantskap tussen hierdie isolate.

'n Studie van homotallisme en die moontlikheid van geslagtelike hibridisering in Colombiaanse *C. cubensis* isolate (Hoofstuk III), het aan die lig gebring dat die fungus homotallies is. Dit is bewys deur enkel-askospoor isolate toe te laat om geslagtelik voort te plant op *Eucalyptus* takkies, gevolg deur genetiese analise met behulp van DNA vingerafdrukke van die nageslag. Die DNA vingerafdruk profiele van die nageslag was identies, met die gevolg dat geen hibridisering plaasgevind het nie. Die geslagtelike gebeurtenis was dus as gevolg van selfbevrugting. Andersyds, tydens genetiese analise van nageslag uit natuurlike perithecia, deur middel van "vegetative compatibility groups" (VCGs) en DNA vingerafdrukke, is gevind dat geslagtelike hibridisering plaasgevind het. *C. cubensis* in Colombië is gevolglik homotallies, maar hibridisering geniet voorrang.

Die genotipiese diversiteit van die Colombiaanse *C. cubensis* populasie is ondersoek met VCGs en RAPDs. Resultate het aangedui dat die genotipiese diversiteit van die populasie ooreenstem met die diversiteit van ander Suid-Amerikaanse populasies van *C. cubensis*. Die fenotipiese (VCG) en genotipiese (RAPD) data vir die Colombiaanse populasie was egter betekenisvol verskillend. Die geskatte waarde vir genotipiese diversiteit gebaseer op RAPDs was aanduidend hoër as dieselfde waarde vir VCG data. Die rede vir die veskil in geskatte waardes word toegeskryf aan die lae sensitiwiteit van VCGs, om genetiese verskille tussen isolate op te spoor.

In Hoofstuk V word 'n nuwe tegniek vir die generasie van polimorfiese mikrosatelliet-agtige DNA merkers uit fungi, beskryf. Die tegniek is gebaseer op die identifikasie en karakterisering van polimorfiese DNA fragmente uit 'n lukrake amplifisering van mikrosatelliet volgordes, deur die PCR (polimerase kettingreaksie) te gebruik.

Basispaaropeenvolgings van hierdie fragmente is gebruik om spesifieke priemstukke te kunstrueer wat sal lei tot amplifisering van polimorfiese lokusse uit genomiese DNA. Die tegniek het 'n hoë sukses-tempo in vergelyking met tradisionele tegnieke vir die isolasie van polimorfiese merkers. Dit is ook bewys dat merkers wat met die nuwe tegniek berei is, gebruik kan word om te onderskei tussen *C. cubensis* isolate afkomstig van Suid-Afrika en ander lande.

Die laaste hooftuk (VI) is 'n beskrywing van die eerste in-diepte molekulêre populasie-studie van *C. cubensis* in Suid-Afrika. Deur polimorfiese merkers uit die vorige studie (Hoofstuk V) te gebruik, was dit moontlik om die molekulêre variansie van die Suid-Afrikaanse populasie te bepaal, en hierdie resultate te vergelyk met fenotipiese data wat voorheen verkry is. Dit is gevind dat molekulêre en fenotipiese data verskillend is met betrekking tot skatting van populasie-diversiteit. 'n Skatting van die gametiese disekwilibrium van die Suid-Afrikaanse *C. cubensis* populasie het aan die lig gebring dat die getoetsde allele lukraak geassosieer is. So 'n geval word verwag van populasies waar geslagtelike voortplanting en die daaropvolgende hibridisering, volop is. Hierdie data is egter 'n indirekte aanduiding dat hibridisering in die Suid-Afrikaanse populasie plaasvind. Toekomstige studies van die Suid-Afrikaanse *C. cubensis* populasie moet gebaseer word op 'n groter aantal merkers, en behoort ook meer isolate in te sluit.

Kennis wat uit hierdie studies ingewin is sal hopelik help om meer effektiewe beheermaatreëls neer te lê om Cryphonectria kanker van *Eucalyptus* bome te bekamp. Nuwe vrae oor die biologie van *C. cubensis* wat dringend ondersoek moet word, het

egter opgeduik. Data uit hierdie tesis kan dus belangrik geag word vir toekomstige studies van die fungus.

ACKNOWLEDGMENTS

I wish to thank the following persons and organisations:

- The Tree Pathology Co-operative Programme and the Forestry and Agricultural Biotechnology Institute for providing facilities and financial support to attend one national and two international conferences during the course of this degree.

- The National Research Foundation and University of Pretoria for financial assistance.

- Prof. Mike Wingfield for collection of *Cryphonectria cubensis* isolates in Colombia.

- All of the previous workers who contributed to a better understanding of fungal pathogens of forest trees, especially *C. cubensis*.

- Proff. Brenda Wingfield, Mike Wingfield and Michael Milgroom for critical review of manuscripts, valuable advice and support.

- Emma Steenkamp, Cassi Myburg and Martin Coetzee for their assistance throughout my studies, critical review of manuscripts and valuable advice.

- God Almighty