Genetic diversity of *Chrysoporthe cubensis* in eastern and southern Africa

Grace Nakabonge[a], Marieka Gryzenhout[a], Brenda D. Wingfield[b] Michael J. Wingfield[a] and Jolanda Roux[a*]

***Chrysoporthe cubensis* is an important fungal pathogen of *Eucalyptus* species worldwide. The fungus is also known on many other hosts, all residing in the order *Myrtales*. Previous studies have suggested that *Chr. cubensis* might be native to South America and southeast Asia and that it has been introduced into Africa. Recently, surveys have been conducted in eastern and southern Africa to assess the distribution of *Chrysoporthe* spp. in this region. *Chr. cubensis* was found on *Eucalyptus* spp. in Kenya, Malawi and Mozambique. The aim of the study reported here was to determine the genetic diversity of *Chr. cubensis* populations from these countries. Population diversity studies were conducted using five pairs of microsatellite markers previously developed for *Chr. cubensis*. Results show that there is a very low genetic diversity within the populations of *Chr. cubensis* from Kenya, Malawi and Mozambique, implying that the fungus was probably recently introduced in these countries. Based on phylogenetic analyses, the origin of East African *Chr. cubensis* is most likely Asia.**

[a]Department of Microbiology and Plant Pathology, and [b]Department of Genetics, DST/NRF Centre of Excellence in Tree Health Biotechnology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.

*Author for correspondence. E-mail: jolanda.roux@fabi.up.ac.za

Introduction

Chrysoporthe cubensis (Bruner) Gryzenh. & M. J. Wingf., previously known as *Cryphonectria cubensis* (Bruner) Hodges,[1] is a fungal pathogen of *Eucalyptus* species in tropical and subtropical areas worldwide.[2] The canker disease caused by *Chr. cubensis* is characterized by the formation of stem cankers, wilting and death of trees.[2–4] The disease is common on *Eucalyptus* spp. in areas with high temperatures and rainfall[3–5] such as South America,[3] Central and North America,[6] Asia[4,5,7] and Africa.[8,9] The fungus also occurs in Australia.[10] Cankers are generally found at the bases of trees, but are often also observed higher up on the stems.[3,4,9] Management of the disease is most typically achieved by planting resistant hybrids and clones.[2,4,11,12]

In Africa, *Chr. cubensis* has been identified on *Eucalyptus* spp. since the 1950s. The fungus is known from the Democratic Republic of Congo (Zaire), where it was thought to be *Cryphonectria havanensis* (Bruner) M.E. Barr,[8] but later identified as *Chr. cubensis*.[13] *Chr. cubensis* is also known from Cameroon[8] and the Republic of Congo (Congo Brazzaville), on *E. grandis* and *E. urophylla* S.T. Blake.[9,14]

Table 1. Isolates of *Chrysoporthe cubensis* used in this study.

Isolate numbers (CMW)[a]	Origin	Host	Collector
13941, 13942, 13945–13947,13951, 13953, 13955, 13956, 14412	Kenya	*Eucalyptus urophylla*	J. Roux
13943, 13944, 13948–13950, 13954, 14411,14757–14765, 14767–14774, 17095–17097, 17099, 17101, 17103, 17104, 17106, 17111, 17113, 17114, 17116, 17117, 17119, 17120–17130, 17134, 17135	Malawi	*E. grandis*	J. Roux
13880, 13883, 13912, 13915, 13920, 13928, 17064, 17071, 17073	Mozambique	*E. grandis*	J. Roux

[a]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

The host range of *Chr. cubensis* is restricted to plants in the order *Myrtales*. In the family *Myrtaceae*, apart from *Eucalyptus* spp., *Chr. cubensis* has been found on *Syzygium aromaticum* (L.) Merry & Perry (Clove) in Indonesia,[15] Sulawesi,[16] Malaysia[17] and Zanzibar.[18] In the family *Melastomataceae*, the fungus has been reported from Colombia on indigenous *Miconia theaezans* (Bonpl.) Cogn. and *M. rubiginosa* (Bonpl.) DC., in Mexico on native *Clidemia sericea* D. Don and *Rhynchanthera mexicana* DC.,[19] in Singapore and Thailand on non-native *Tibouchina urvilleana* (DC). Logn. and in Indonesia on native *Melastoma malabathricum* L.[19] Recently, *Chr. cubensis* has also been reported from Cuba on the non-native *Lagerstroemia indica* L. (*Lythraceae*),[19] and reports have been made from three native hosts in Brazil.[20,21] These represent the first records of the fungus on a plant family other than the *Myrtaceae* and *Melastomataceae*.

Until recently, *Chrysoporthe* spp. were treated under the single name *C. cubensis,* responsible for a disease known collectively as Cryphonectria canker.[1] Morphological characteristics and phylogenetic analysis of DNA sequence data of the histone H3 gene and β-tubulin 1&2 gene regions have revealed that within *C. cubensis* there are distinct phylogenetic groups.[1,22,23] The South African group includes isolates that have been described as *Chr. austroafricana* Gryzenh. & M.J. Wingf.[1] Another group comprises the American (Central, North and South) and West and Central African isolates (South American group). A third group includes the southeast Asian, East African and Australian isolates (southeast Asian group).[14,23,24] The latter two groups are phylogenetically different but they are treated as *Chr. cubensis,* since no distinct morphological differences have been observed between them.[1]

There are two hypotheses regarding the possible origin of *Chr. cubensis*. One view is that the fungus is native on indigenous clove trees (*S. aromaticum*) in the Molucca Islands of Indonesia and that it spread to other areas, possibly with the spice trade.[15] This view of the origin of *Chr. cubensis* is supported by the high genetic diversity revealed using Vegetative Compatibility Groups (VCGs) within the Indonesian population of the pathogen.[25] An alternative hypothesis is that *Chr. cubensis* originated from South America.[2,26] This is supported by the high genetic diversity revealed using VCGs within the Venezuelan population of *Chr. cubensis.*[25] Studies conducted on Brazilian isolates by Van Zyl *et al.*[27] also revealed a high diversity in that region. The recent discovery of *Chr. cubensis* on native plants in Colombia[28] and Mexico[19] supports the view that the fungus has a South American origin. However, if the pathogen represents two distinct species as phylogenetic data suggest,[1] native populations of *Ch. cubensis* would logically occur in both southeast Asia and in South America.[19]

Several disease surveys have been conducted in southern and eastern Africa to assess the distribution of *Chrysoporthe* spp. in the region.[24,29] *Chr. cubensis* was subsequently reported for the first time from Kenya, Malawi and Mozambique. All the isolates from these areas were shown to group in the southeast Asian clade of *Chr. cubensis*.[24] The aim of the present study was to assess the level of genetic variability in Kenyan, Malawian and Mozambican populations of *Chr. cubensis.* This would provide information on the possible origin of this species, which has been postulated to have been introduced into Africa.[1,24]

Materials and methods

Fungal isolates

Pure cultures of *Chr. cubensis* used in this study were those from collections made by Roux *et al.*[29] and identified by Nakabonge *et al.*[24] Fifty-one isolates were obtained from *Eucalyptus* trees growing in two adjacent plantations in Malawi, ten isolates from *E. urophylla* in a single plantation in Kenya, and nine isolates from the Manica area in Mozambique (Table 1). All isolates used in this study are housed in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Isolates were grown in Petri plates on 2% malt extract agar (MEA) (20 g/l malt extract and 15 g/l agar, Biolab, Midrand, South Africa). To reduce the potential for bacterial contamination, 100 mg streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany) was added to the MEA, after which cultures were incubated at 26°C for seven days. Mycelium was scraped from the plates, transferred to 1.5-ml Eppendorf tubes and DNA extracted as previously described by Myburg *et al.*[22]

Simple sequence repeat PCR

Eight pairs of PCR-based simple sequence repeat (SSR) primers developed by Van der Merwe *et al.*[30] for *Chr. cubensis* were tested on the African isolates. The PCR reaction mixes and conditions were the same as those described by Van der Merwe *et al.*[30] The DNA concentrations of the PCR products were visually measured against the intensity of a 100-bp marker (Roche Molecular Biochemicals, Mannheim, Germany) on a 2% agarose gel stained with ethidium bromide and exposed to UV illumination.

PCR products were diluted for Genescan analysis based on the approximate concentrations of the PCR products. Samples were separated on a 4.25% PAGE gel, using an ABI Prism™ 377 DNA sequencer (Applied Biosystems, Foster City, CA). Allele sizes were estimated by comparing the mobility of the SSR products with that of the TAMRA internal size standard (Applied Biosystems, Perkin Elmer Corp.) as determined by Genescan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 2 (Applied Biosystems). To ensure reproducibility, a reference sample was run on every gel.

Genetic diversity and differentiation

Isolates were scored based on allele size at each locus. This information was used to generate a multilocus profile or genotype for each isolate. Identical genotypes were treated as clones and statistics were calculated for clone-corrected populations. Allele frequencies in each population were then calculated by dividing the number of times an allele occurred in the population by the population sample size. The allele frequencies were used to calculate the gene diversity,[31] $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the kth allele, for each population using the program POPGENE version 1.31.[32] Differences in allele frequencies for clone-corrected populations were estimated by calculating chi-squared tests (χ^2).[33]

Genotypic diversity was calculated using the formula $G = 1/\sum [f_x (x/n)^2]$, where n is the sample size and f_x is the number of genotypes (haplotypes) occurring x times in the population; G is the effective number of equally frequent haplotypes.[34] The genotypic diversities between populations were compared by obtaining the maximum percentage of

Table 2. Allele size (bp) and frequency at 5 loci for clone-corrected populations of *Chr. cubensis* from *Eucalyptus* spp. in Kenya and Malawi.

Locus	Allele length	Allele frequencies	
		Kenya	Malawi
SA6	206	–	0.14
	210	1.0	0.71
	245	–	0.14
SA9	194	0.5	1.00
	195	0.5	–
	196	–	–
COL3	167	–	0.28
	176	1.0	0.71
	177	–	–
SA10	181	–	0.14
	182	1.0	0.42
	183	–	0.42
SA1	319	1.0	1.00
	–	–	–
	–	–	–
N(g)		2	9
N		10	51
Number of alleles		7	10
G		1.72	2.75
$\hat{G}$ (%)		17.2	5.4

N, number of isolates; *N*(*g*), number of multilocus genotypes; *G*, genotypic diversity[34]; $\hat{G}$, percentage maximum diversity

genotypic diversity using the formula $\hat{G} = G/N*100$, where N is the sample size.[35]

Genetic distance

The genetic distance was calculated between *Chr. cubensis* genotypes based on Nei's[36] unbiased genetic distance. The distance matrix was generated using the program POPGENE version 1.31 and a tree constructed using UPGMA (Unweighted Pair–Group Method with Arithmetic mean) in MEGA version 2.1.[37]

Results

Simple sequence repeat PCR

Five of the eight pairs of PCR-based SSR primers (SA6, SA9, COL3, SA10, SA1) amplified the microsatellite regions for the African isolates. Allele sizes were successfully estimated for all microsatellite regions (Table 2).

Genetic diversity and differentiation

In total, seven alleles were amplified across the five loci for the *Chr. cubensis* population from Kenya and 10 alleles for the population from Malawi (Table 2). Isolates from Mozambique were not included in the analysis because they all belonged to a single clone that occurred in both the Kenyan and Malawian populations. Locus SA1 was monomorphic in both populations. The remaining four loci had a total of three alleles each. Six alleles were shared between populations. Thus, there was a total of seven unique alleles. The χ^2 tests for the five microsatellite regions showed no significant difference in allele frequencies at any loci, between the Kenyan and Malawian populations of *Chr. cubensis* (Table 3).

A total of nine genotypes was obtained when the *Chr. cubensis* populations were combined. One genotype was shared between the Kenyan and Malawian populations, giving a total of 8 unique genotypes. The maximum genotypic diversity was 17.2% for the Kenyan and 5.4% for the Malawian population (Table 2).

Genetic distance

The UPGMA tree constructed from the matrix obtained using Nei's[36] genetic distance clearly showed that there was no grouping of isolates according to the areas sampled (Fig. 1). Different genotypes were equally distributed throughout the populations sampled.

Table 3. Gene diversity (*H*) and contingency χ^2 tests for differences in allele frequencies* for the five polymorphic SSR loci across clone-corrected populations of *Chr. cubensis* collected from *Eucalyptus* spp. in Kenya and Malawi.

Locus	Kenya	Malawi	χ^2	d.f.
SA6	0	0.44	0.73	2
SA9	0.5	0.00	3.9	1
Col3	0	0.40	0.73	1
SA10	0	0.61	2.0	2
SA1	0	0.00	0	0
N	2	7		
Mean	0.1	0.29		

*There were no significant differences between allele frequencies at any loci ($P < 0.005$).

Discussion

The population structure of a collection of *Chr. cubensis* isolates from *Eucalyptus* spp. in Kenya, Malawi and Mozambique was considered for the first time in this study. This represents the first attempt to consider the genetic structure of the fungus from eastern and southern Africa. The low genetic diversity in combination with phylogenetic data[1,23,24] suggests that *Chr. cubensis* from eastern and southern Africa did not originate in Africa.

Newly introduced populations are expected to possess lower diversities and very few private alleles, compared with those of native populations.[38,39] Very low genetic diversities were observed within the Kenyan and Malawian populations and only a single clone of the fungus was found in Mozambique. Genetic migration was also observed between isolates representing the Kenyan and Malawian populations. There were no significant differences between chi-square values at any of the loci in either population. Such low measures of diversity strongly suggest that *Chr. cubensis* has been introduced into these countries relatively recently.

Processes such as mutation that result in increased diversity in a population, occur in well-established populations.[38,39] The results obtained in this study imply that there has not been

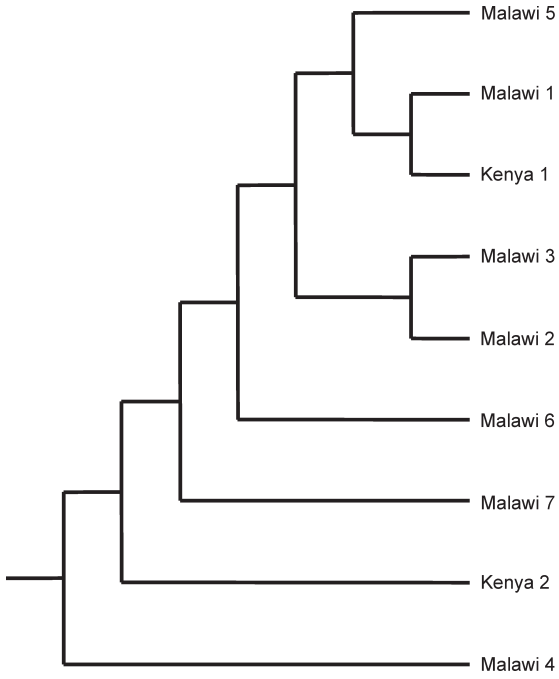

Fig. 1. UPGMA dendrogram of *Chr. cubensis* genotypes from Malawi and Kenya, collected from *Eucalyptus* spp., constructed with clone-corrected data obtained using five polymorphic microsatellite markers.

sufficient time to allow such processes to occur in the African populations of *Chr. cubensis.* However, results of this study should be viewed as preliminary and larger numbers of isolates are required from Mozambique before definitive conclusions can be derived for this region. Yet, the fact that the Mozambican isolates examined are clonal, identical to a clone that occurs in both Malawi and Kenya, and that the fungus is found rarely in the country (of a collection of 89 *Chrysoporthe* spp. collected from Mozambique, only nine isolates proved to be *Chr. cubensis*; unpublished data) suggest that the fungus has been introduced recently.

Chrysoporthe cubensis has never been reported on a native host in Africa. This makes the continent a highly unlikely centre of origin for the fungus. The pathogen is also not known to occur in South Africa, where *Eucalyptus* hybrids and clones are widely grown in plantations. However, the occurrence of *Chr. cubensis* in bordering Mozambique is threatening to an important *Eucalyptus* plantation industry in this country. Every effort must thus be made to slow the advance of *Chr. cubensis* into South Africa and to start screening South African *Eucalyptus* planting stock for tolerance to this pathogen. The fact that the populations considered in this study are genetically uniform could imply that management strategies to reduce the impact of the disease in eastern and southern Africa have a good chance of succeeding.

We acknowledge financial support provided by the members of the Tree Protection Co-operative Programme, the THRIP initiative of the Department of Trade and Industry, the DST/NRF Centre of Excellence in Tree Health Biotechnology, South Africa; the Third World Organization for Women in Science (TWOWS), Makerere University, Uganda; and the British Society for Plant Pathology. We thank the following persons and institutions in the different countries for assistance during sampling: L. Mwangi of the Kenyan Forestry Research Institute; W. Sagona of the Malawian Forestry Research Institute; C. Nguvulu of the Zambian Forestry Research Institute; E. da Cruz and I.F. Maluleque of Centro de Experimentação Florestal, Mozambique, and P. Swanepoel of Ifloma, Mozambique.

Received 27 February. Accepted 13 June 2007.

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