Identification of *Cryphonectria cubensis* from Colombia based on rDNA sequence data

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CRYPHONECTRIA CUBENSIS IS A PATHOGEN of *Eucalyptus* trees in tropical and subtropical regions of the world. The fungus causes a disease known as Cryphonectria canker, which ultimately results in the death of trees. Isolates of *C. cubensis* are usually identified using a combination of morphological and molecular characters, such as ascospore morphology and ITS (internal transcribed spacer) ribosomal DNA sequence data. Using these techniques, the fungus has been identified from Brazil, Venezuela, South Africa, Indonesia, Australia and several other countries. In this study, we investigated isolates of *C. cubensis* from Colombia and report for the first time the presence of *C. cubensis* west of the Andes in South America.

Cryphonectria cubensis is the causal agent of Cryphonectria canker, which is a disease characterized by girdling cankers on the stems of *Eucalyptus* trees.[1-3] This disease is a significant threat to commercial *Eucalyptus* plantations in tropical and subtropical countries, since severe infection can lead to stem damage and tree death.[3-5] Recent research on the disease has, therefore, focused on elucidating pathogenicity patterns,[5,6] population diversity,[7,8] and the phylogenetic relationships[9] of *C. cubensis* from different geographical areas.

The phylogenetic relationships between isolates of *C. cubensis* originating in South Africa, South America, Asia and Australia have recently been elucidated.[9] This study involved phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) operon. Results indicated that *C. cubensis* represents a monophyletic group. Within this group, however, two separate clades can be discriminated. Isolates from South America and South Africa represent a clade separate from those originating in South East Asia. It was thus postulated that *C. cubensis* has probably been introduced into South Africa from South America.[9]

C. cubensis has recently been identified as a serious pathogen in Colombia. Nothing is known regarding the taxonomy of this fungus, whose presence is now also recognized for the first time west of the Andes in South America. The aim of the study was, therefore, to identify the fungus conclusively and to consider the phylogenetic relatedness of Colombian *C. cubensis* isolates to those from other parts of the world.

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Materials and methods

Morphology: bark pieces from *Eucalyptus* trees in Colombia were incubated in moist Petri dishes at 26°C for 2 days. Perithecia, asci and ascospores were then studied using light microscopy, and features were compared to those previously described for *C. cubensis*.[2,11]

Fungal isolates and DNA extraction: genomic DNA was extracted from four isolates (CRY1209, CRY1242, CRY1318 and CRY1394) of *C. cubensis* from Colombia, using the protocol described by Raeder and Broda.[12] All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

PCR amplification: the ITS1, 5·8S and ITS2 regions of the rDNA operon were amplified using primers ITS1 and ITS4[13] with 25 ng of genomic DNA from each of the four Colombian *C. cubensis* isolates. The reactions were then carried out as described by Myburg *et al.*[9] Ten microlitres of each PCR amplification product were electrophoresed (6 V/cm for 1 h 30 min) on a 1% w/v agarose gel containing 0.05 μg/ml ethidium bromide and visualised using ultraviolet light, to confirm the presence of a single band of *c.* 500 bp (base pairs).

Sequencing and data analyses: PCR amplification products were purified using a Nucleospin Extract PCR Purification Kit (Macherey Nagel) and sequenced using a Dye Terminator Cycle Sequencing Kit (Perkin Elmer). Electropherograms were analysed using the Sequence Navigator computer software, followed by manual alignment with previously published sequences from *C. parasitica* and *D. ambigua*.[9] Aligned sequences were subjected to phylogenetic analyses using parsimony (PAUP* 4.0).[14] The sequence for *D. ambigua* was used as outgroup with a heuristic search using TBR (tree bisection reconnection) of the data set. Bootstrap analysis (1000 replicates) was used to determine the confidence interval of each branch. Sequences were deposited in GenBank.

Results

Morphology: morphological features of the Cryphonectria canker fungus from Colombia were identical to those described for isolates of *C. cubensis* from South America.[2] Perithecia were thus abundant on cankers and these contained typical asci and ascospores. Pycnidia were not observed.

PCR amplification: amplification of the ITS rDNA region using PCR yielded a *c.* 500-bp 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 amplification products resulted in a readable sequence of *c.* 500 bp for all four Colombian *C. cubensis* isolates. Manual alignment of ITS sequence data resulted in a total of 577 characters.

Heuristic analysis of the 577 aligned characters generated one most parsimonious tree (Fig. 1). Three clades were identified from the phylogenetic tree. One clade comprised three *C. parasitica* isolates and 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, in close proximity to each other, are present in the ITS2 region of the rRNA operon. These sequences occur at characters 452, 459–460, and 486, respectively. 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.

Discussion

In this study, it was confirmed that recently collected isolates from *Eucalyptus* cankers in Colombia are typical of *C. cubensis*. This is the first definitive record of the fungus west of the Andes in South America. The isolates are also phylogenetically closely related to other isolates of the fungus from South America. This finding suggests that the South American

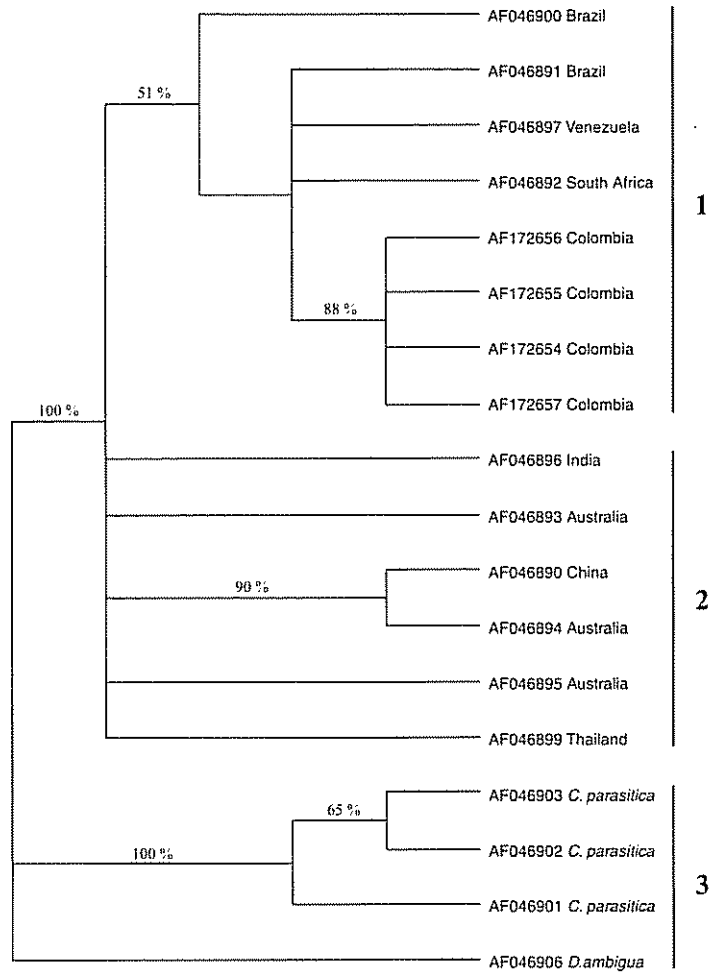

Fig. 1. A rectangular cladogram generated after a heuristic search of the ITS sequence data set (tree length = 261; CI = 0 985; RI = 0 986). Bootstrap values are indicated on each branch. The origin of each isolate is indicated after the Genbank accession number. Clade 1 includes South American isolates, clade 2 represents isolates from South East Asia, and *C. parasitica* isolates are included in clade 3, together with the outgroup taxon, *D. ambigua*. Clades 1 and 2 can be unambiguously distinguished from each other by three signature sequences in the aligned data set.

C. cubensis isolates had a common origin, which is consistent with the findings of Myburg *et al.*[9]

Phylogenetic analysis supported the grouping of *C. cubensis* isolates into two distinct clades, namely a South American and a South East Asian clade.[9] Based on ITS DNA sequences, therefore, 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 more closely related to each other than they are to isolates from South East Asia.

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, and would be useful in the development of diagnostic techniques to distinguish between *C. cubensis* isolates from different countries. Myburg *et al.*[9] devised 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 geographical origins, using differential primers during PCR amplification.

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 America. This finding suggests that *C. cubensis* in Colombia might display the same population level properties as the fungus in Venezuela[15] and Brazil,[16] which will have an impact on studies currently underway to develop *Eucalyptus* planting stock that is tolerant to Cryphonectria canker.

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S_2A_3 Centenary in 2002

The Southern Africa Association for the Advancement of Science celebrates its centenary next year. Members who wish to do so are invited to send the National Secretary, Shirley Korsman, their recollections and reminiscences, especially of the early days of S_2A_3. Experiences, stories of friendships, anecdotes, tributes to scientists, memories of conferences — in fact, anything that would be of interest to S_2A_3 today is welcome.

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