PRIMER NOTE

Primers for the amplification of sequence-characterized loci in *Cryphonectria cubensis* populations

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Abstract

We describe the development of DNA markers for the fungal pathogen of *Eucalyptus*, *Cryphonectria cubensis*. These markers originated from cloned intershort sequence repeat polymerase chain reactions, which enrich for medium to highly repetitive DNA sequences. In total, 10 markers were isolated, eight of which were polymorphic, and these can subsequently be applied to study populations of *C. cubensis*.

Keywords: co-dominant markers, ISSR-PCR, moderately repetitive sequences, populations, primer sets

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*Cryphonectria cubensis* causes a debilitating stem canker disease on *Eucalyptus* trees in many countries of South America (Hodgès et al. 1979; Van Zyl et al. 1998), Africa (Wingfield et al. 1989) and Southeast Asia (Davison & Coates 1991). Vegetative compatibility groups have been used to study some populations of this pathogen to consider questions of origin and local genetic diversity (Van Zyl et al. 1994; Van Heerden et al. 1997; Wingfield et al. 1997; Van Zyl et al. 1998). This technique makes possible the identification of individual phenotypes that are capable of hyphal anastomosis at any point of contact (Newhouse & MacDonald 1991; Leslie 1993). Screening is thus for genotypes based on an unknown number of loci. Although the technique has many advantages, lack of resolution precludes the possibility of deducing population parameters such as genetic diversity, outcrossing rates and migration patterns (Milgroom et al. 1993; Cortesi et al. 1996). The aim of the present study was therefore to develop co-dominant DNA markers that would make it possible to analyse populations of *C. cubensis* at a considerably higher level of resolution than was previously possible.

Genomic DNA was extracted from three *C. cubensis* isolates (CMW6112, CMW8856 and CMW8890) using a CTAB extraction protocol (Murray & Thompson 1980). An intershort sequence repeat (ISSR) polymerase chain reaction (PCR) was performed on extracted DNA, using four different ISSR primers (Table 1). All ISSR PCR reactions were performed using the protocol of Hantula et al. (1996). Amplification products were cloned into the pGEMT-Easy plasmid (Promega Corporation) transformed into and propagated in *Escherichia coli* JM109 cells. Plasmids were extracted and purified (Sambrook et al. 1989) and insert sizes were determined using a restriction digest with *Eco*<sub>R</sub> followed by agarose gel electrophoresis. Inserts of different sizes and smaller than 600 base pairs (bp), were cycle sequenced using the BigDye Dye Terminator Kit (Perkin Elmer) with M13 primers SP6 and T7, followed by electrophoresis using an ABI Prism 377 automated sequencing instrument. Specific primers were designed to flank medium repetitive sequences present in several inserts, and were used to amplify the loci from genomic DNA of the original *C. cubensis* isolates from South Africa and Mexico, as well as isolates from Colombia, Republic of Congo, Indonesia and Vietnam. Each 25-µL PCR reaction contained 1 ng/µL genomic DNA, 10 mM MgCl<sub>2</sub>, 2.5 mM of each dNTP, 1 × PCR buffer (Southern Cross Biotechnology), 0.8 mM 2-pyrrolidinone (Chakrabarti and Schutt 2001), 40 µM of each primer, and 1 U SuperTherm *Taq* Polymerase (Southern Cross Biotechnology). PCR was performed on a GeneAmp® PCR System 9700 (Applied Biosystems), and was initiated with denaturation at 94 °C for 2 min, followed by 30 cycles of 54 °C for 30 s, 72 °C for 1 min and 94 °C for 1 min. PCR was completed with a final extension at 72 °C for 7 min.

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Sequences of inserts from which primers were designed were used in BLAST searches of the GenBank database (Altschul et al. 1990), to determine their similarity to known sequences. Two separate searches were performed for each sequence: (i) a BLAST search restricted to the fungi and (ii) a BLAST search restricted to Saccharomyces cerevisiae.

After agarose gel electrophoresis (1% w/v) and visualisation using ethidium bromide and ultraviolet light, PCR products from isolates CMW6112 and CMW8856 with primers (CGA)_5 and (CAO)_5 revealed easily distinguishable fingerprints. Primer (GT) yielded unique fingerprints for CMW6112 and CMW8890, but primer (CCA)_5 did not provide unique fingerprints for the different isolates. After cloning the PCR amplification products into the plasmid, 307 recombinant colonies were recovered. Restriction digests of purified plasmid DNA revealed that 58 clones contained inserts smaller than 600 bp. Of these, 22 inserts were of different size. Primers were designed from these 22 sequences. Only 10 of the 22 primer pairs consistently resulted in the amplification of the desired targets across all isolates during PCR (Table 1).

GeneScan® analysis of 56 test isolates revealed that four of the candidate loci were monomorphic based on size. These loci, namely COL4, COL6, SA2 and SA10, may still contain sequence polymorphisms not detectable by the GeneScan® technique. The remaining six primer pairs amplified varying numbers of alleles from the 56 test isolates (Table 2) and therefore, varied in their levels of polymorphism. Primer pairs for loci SA6 and SA9 each yielded PCR products from two loci and in both cases these loci were polymorphic.

BLAST searches using the fungal database revealed that sequences for two loci, namely SA1 and SA3, were homologous to a Neurospora crassa sequence (GenBank accession AL356815) (Table 1). A search on the S. cerevisiae database showed that several of the C. cubensis sequences were homologous to sequences present on different chromosomes of the yeast. Five of the C. cubensis sequences showed weak homology to sequences of S. cerevisiae chromosome XV.

In this study, we have succeeded in developing 10 primer sets that amplify loci from C. cubensis isolates originating in Colombia, Mexico, Indonesia, Vietnam, South Africa and the Republic of Congo. Six of these primer sets could be used to amplify polymorphic loci from genomic DNA of C. cubensis, while four were unsuccessful in targeting

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**Table 1** Primer sequences, their origins and amplicon homologies to sequences deposited in GenBank

<table>
<thead>
<tr>
<th>Source isolate*</th>
<th>ISSR primer</th>
<th>Locus name</th>
<th>Fungal BLAST, most homologous result†</th>
<th>S. cerevisiae BLAST most homologous chromosome (E-value)</th>
<th>Primer sequence (5’ → 3’)‡</th>
<th>GenBank accession no.</th>
<th>Amplicon size (bp)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMW8856</td>
<td>(CGA)_5</td>
<td>COL6</td>
<td>AF246264 (1.4)</td>
<td>XIV (5.6)</td>
<td>F GGCCAGCGCGACGTGCAAAGCG</td>
<td>AY280941</td>
<td>242</td>
</tr>
<tr>
<td>CMW8890</td>
<td>(GT)_5</td>
<td>COL7</td>
<td>AJ009934 (1.3)</td>
<td>XVI (1.3)</td>
<td>R GTTCTGGACAGTCGCGCGCG</td>
<td>AY280942</td>
<td>175</td>
</tr>
<tr>
<td>CMW8890</td>
<td>(GT)_5</td>
<td>COL11</td>
<td>AL670003 (0.3)</td>
<td>XVI (4.6)</td>
<td>F GTTATCGGCATCTCTGTCG</td>
<td>AY280943</td>
<td>262</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(CAC)_5</td>
<td>SA1</td>
<td>AF004553 (0.054)</td>
<td>VIII (0.83)</td>
<td>F GCAGATCCACTAGCTAGGCTC</td>
<td>AY280944</td>
<td>320</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(CAC)_5</td>
<td>SA3</td>
<td>AL356324 (0.054)</td>
<td>VIII (0.82)</td>
<td>R CTGTCTCCCTTAAAGGTGATG</td>
<td>AY280945</td>
<td>207</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(CGA)_5</td>
<td>SA4</td>
<td>AF281307 (0.012)</td>
<td>X (2.9)</td>
<td>R CTGAGCAGCCACTAGCTAGT</td>
<td>AY280946</td>
<td>163</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(GT)_5</td>
<td>SA6</td>
<td>AF107791 (0.37)</td>
<td>V (0.36)</td>
<td>R GTTATCGGCATCTCTGTCG</td>
<td>AY280947</td>
<td>208</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(CAC)_5</td>
<td>SA7</td>
<td>AL669986 (0.007)</td>
<td>I (0.11)</td>
<td>F CTGAGATGATGAATCAGT</td>
<td>AY280948</td>
<td>232</td>
</tr>
<tr>
<td>CMW6112</td>
<td>(CAC)_5</td>
<td>SA9</td>
<td>AL669986 (0.005)</td>
<td>I (0.073)</td>
<td>F CTTGCCAGCAAGCTCTGTC</td>
<td>AY280949</td>
<td>194</td>
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<tr>
<td>CMW6112</td>
<td>(CAC)_5</td>
<td>SA10</td>
<td>AJ295347 (0.03)</td>
<td>V (0.03)</td>
<td>F GCCAGGATCGCTCTGAGAG</td>
<td>AY280950</td>
<td>184</td>
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</table>

*All fungal isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
†All results are given as GenBank accession numbers followed by the E-value of the GenBank match.
‡‘F’ and ‘R’ denote forward and reverse primers, respectively.
§Amplicon sizes were experimentally verified using PCR with genomic DNA from the source isolates, and electrophoresed on a polyacrylamide gel.

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Table 2 Allele frequencies for polymorphic markers developed during this study, and tested on 56 isolates of *Cryphonectria cubensis*

<table>
<thead>
<tr>
<th>Markers</th>
<th>Alleles</th>
<th>Occurrence</th>
<th>Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA1</td>
<td>312</td>
<td>1</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>319</td>
<td>42</td>
<td>0.750</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>9</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>null</td>
<td>4</td>
<td>0.071</td>
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<tr>
<td>SA4</td>
<td>160</td>
<td>1</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>164</td>
<td>17</td>
<td>0.304</td>
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<td></td>
<td>166</td>
<td>38</td>
<td>0.679</td>
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<tr>
<td>SA6, Locus 1</td>
<td>203</td>
<td>8</td>
<td>0.143</td>
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<tr>
<td></td>
<td>204</td>
<td>4</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>205</td>
<td>10</td>
<td>0.179</td>
</tr>
<tr>
<td></td>
<td>206</td>
<td>10</td>
<td>0.179</td>
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<td></td>
<td>207</td>
<td>3</td>
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<td></td>
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<td>33</td>
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<td></td>
<td>214</td>
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<td>SA9, Locus 1</td>
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<td>202</td>
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<tr>
<td></td>
<td>203</td>
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<td>0.018</td>
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<tr>
<td>SA9, Locus 2</td>
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<tr>
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<td>0.018</td>
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<td>8</td>
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</tr>
<tr>
<td>COL3</td>
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<tr>
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<td>6</td>
<td>0.107</td>
</tr>
</tbody>
</table>

Allele names are based on the size of each allele, as determined from Genescan® analysis.

The method followed in this study sufficiently enriches for repetitive sequences to allow quick and easy cloning of markers. Our results are therefore in accordance with results obtained for *Eucalyptus*, using a similar approach (Van der Nest et al. 2000).

Two of the loci (SA7 and SA9), in *C. cubensis* showed significant similarity to a single sequence of *N. crassa*, when a BLAST search was performed on the fungal database. This, together with the fact that both of these sequences also show similarity to *S. cerevisiae* chromosome I, indicates that SA7 and SA9 are probably in close proximity to each other in the *C. cubensis* genome. The lack of a genetic map for the *C. cubensis* genome, however, makes it impossible to test this hypothesis.

The markers developed in this study will provide a robust tool for future population studies of *C. cubensis*. These will have a positive impact on the *Eucalyptus* breeding programmes currently being developed, by reducing the impact of Cryphonectria canker. These markers will also be useful in studies that are currently underway to determine the origin and international movement of *C. cubensis*.

Acknowledgements

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References


