Coffee berry disease pathogen in Africa: genetic structure and relationship to the group species *Colletotrichum gloeosporioides*

S. SREENIVASAPRASAD1, AVERIL E. BROWN1,2* AND P. R. MILLS1,2

Restriction fragment length polymorphisms of the ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of isolates of the coffee berry disease pathogen in Africa, *Colletotrichum kahawae*, were analysed using rDNA from *Saccharomyces cerevisiae* and mtDNA extracted from *C. kahawae* and *C. gloeosporioides* as probes. These analyses revealed homogeneity among *C. kahawae* isolates. The estimated sizes of the rDNA repeat unit and the mtDNA of *C. kahawae* were 8.4–9.18 kb and 60 kb, respectively. Polymorphisms were observed in rDNA and mtDNA when *C. kahawae* was compared with *C. gloeosporioides* from coffee, avocado and mango. However, an avocado isolate JIA1, from Australia, had an identical rDNA restriction pattern to *C. kahawae* when digested with BamHI and *C. kahawae* showed greater than 96% similarity to two *C. gloeosporioides* avocado isolates (918 and 1072) from New Zealand in mtDNA restriction fragment pattern. Random amplified polymorphic DNA analysis also grouped the *C. kahawae* isolates together. Nucleotide sequence of the internally transcribed spacer 1 region of the rDNA repeat unit of *C. kahawae* and *C. gloeosporioides* isolates from *Coffea* spp. differed by only two to three bases (98.8–98.2% homology). Results obtained confirmed the close genetic relationship of *C. kahawae* to the group species *C. gloeosporioides*.

Coffee berry disease (CBD) is an anthracnose of green and ripe coffee berries recorded from various parts of the African continent (Masaba & Waller, 1992). The CBD pathogen *Colletotrichum kahawae* Waller & Bridge (= *C. coffeicum* Noack) (Waller et al., 1993) is distinguished from *C. gloeosporioides* (Penz.) Penz. & Sacc. by its ability to infect green berries (Waller, 1982) and by cultural and biochemical characteristics (Waller et al., 1993). *C. gloeosporioides* isolates from coffee are either saprobes or pathogenic only on ripe berries and leaves. The CBD pathogen, when newly isolated from the host, is characterized by slow growing, dark grey to olivaceous green profuse mycelium, and setae are rarely present. However, after subculturing several times the colonies of CBD isolates become indistinguishable from the morphologically extremely variable group species *C. gloeosporioides* (Sutton, 1992; Waller et al., 1992). On the basis of this observation, Hocking, Johans & Vermeulen (1967) claimed that the CBD pathogen was 'merely a segregant of *Glomerella cingulata* (Stonem.) Spauld. & Schenk. '. Subsequently, Masaba & Waller (1992) pointed out that, although von Arx (1957) considered the CBD pathogen as a conidial state of *G. cingulata*, evidence for the existence of a perfect state of the CBD pathogen is lacking.

Recently, restriction fragment length polymorphism (RFLP) analyses of ribosomal and mitochondrial DNA, random amplified polymorphic DNA (RAPD) analysis and sequencing of the variable internally transcribed spacer (ITS) 1 region of rDNA have been used to elucidate the molecular variation of isolates of *C. gloeosporioides* and other *Colletotrichum* spp. which infect fruit crops (Braithwaite, Irwin & Manners, 1990; Hodson, Mills & Brown, 1992; Mills, Hodson & Brown, 1992a; Sreenivasaprasad, Brown & Mills, 1992). The present study was undertaken, using these techniques, to determine the genetic structure of the CBD pathogen and its relatedness to the group species *C. gloeosporioides*.

**MATERIALS AND METHODS**

**Fungal isolates and growth conditions**

Isolates of *C. kahawae* and *C. gloeosporioides* used in this study originated from sources shown in Table 1. Of the International Mycological Institute (IMI) isolates, all except IMI 325945 and 325946 displayed cultural characteristics typical of CBD isolates (Waller, 1982; Sutton, 1992). The *C. gloeosporioides* isolates obtained from *Coffea* sp. in Sri Lanka were isolated from anthracnose lesions on ripe coffee berries and single conidium-derived cultures produced. All cultures were maintained on potato dextrose agar (PDA, Oxoid).

To produce mycelium for DNA extraction all isolates were grown for 3 d at 25 °C in an orbital incubator (120 rpm) in Erlenmeyer flasks (250 ml) containing 100 ml liquid medium (11 contained glucose, 10 g; NH4H2PO4, 1 g; KCl, 0.2 g; MgSO4, 7H2O, 0.2 g; yeast extract (Difco), 5 g; 0.5% w/v CuSO4, 5H2O, 1 ml; 1% w/v ZnSO4, 7H2O, 1 ml). The inoculum comprised 8 discs (4 mm diam.) cut from the leading...
Genetic structure of the CBD pathogen

Table 1. Host and country of origin of isolates of the coffee berry disease pathogen Colletotrichum kahawae and of C. gloeosporioides

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Country</th>
</tr>
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<tbody>
<tr>
<td>C. kahawae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMI 299393</td>
<td>Coffea arabica</td>
<td>Cameroon</td>
</tr>
<tr>
<td>190857</td>
<td>Coffea arabica</td>
<td>Ethiopia</td>
</tr>
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<td>319406</td>
<td>Coffea arabica</td>
<td>Kenya</td>
</tr>
<tr>
<td>319418</td>
<td>Coffea arabica</td>
<td>Kenya</td>
</tr>
<tr>
<td>344706</td>
<td>Coffea arabica</td>
<td>Kenya</td>
</tr>
<tr>
<td>348838</td>
<td>Coffea sp.</td>
<td>Kenya</td>
</tr>
<tr>
<td>301220</td>
<td>Coffea arabica</td>
<td>Malawi</td>
</tr>
<tr>
<td>338731</td>
<td>Coffea arabica</td>
<td>Malawi</td>
</tr>
<tr>
<td>338734</td>
<td>Coffea arabica</td>
<td>Malawi</td>
</tr>
<tr>
<td>311655</td>
<td>Coffea sp.</td>
<td>Tanzania</td>
</tr>
<tr>
<td>338730</td>
<td>Coffea arabica</td>
<td>Zambia</td>
</tr>
<tr>
<td>300964</td>
<td>Coffea arabica</td>
<td>Zimbabwe</td>
</tr>
<tr>
<td>C. gloeosporioides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMI 325945</td>
<td>Coffea arabica</td>
<td>Malawi</td>
</tr>
<tr>
<td>325946</td>
<td>Coffea arabica</td>
<td>Malawi</td>
</tr>
<tr>
<td>CGcofA</td>
<td>Coffea arabica</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>CGcofC</td>
<td>Coffea sp.</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>CGcofR</td>
<td>Coffea sp.</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>918</td>
<td>Persea sp.</td>
<td>New Zealand</td>
</tr>
<tr>
<td>1072</td>
<td>Persea sp.</td>
<td>New Zealand</td>
</tr>
<tr>
<td>JIA1</td>
<td>Persea sp.</td>
<td>Australia</td>
</tr>
<tr>
<td>M3/7</td>
<td>Mangifera sp.</td>
<td>Sri Lanka</td>
</tr>
</tbody>
</table>

edge of actively growing cultures on PDA. Mycelium was harvested by filtration through Whatman No. 3 filter paper, immediately frozen in liquid N2, pulverized, lyophilized, ground to a fine powder and stored at -70°C.

DNA extraction

Total DNA was extracted from the mycelial powder using the method of Raeder & Broda (1985). Freeze dried mycelial powder (300 mg) was extracted in 3 ml extraction buffer and 3 ml phenol/chloroform (2:1 ml + 0.9 ml). After RNase treatment, two phenol/chloroform extractions were carried out. The DNA, precipitated with 0.54 volumes isopropanol, was dissolved in 400-500 μl 10 mM Tris HCl and 1 mM EDTA solution, pH 8.

Mitochondrial DNA (mtDNA) was isolated from total DNA extracted from 6 g mycelial powder. MtDNA was fractionated on cesium chloride/bis-benzimide gradient (1.68 g ml⁻¹) formed by centrifugation at 40000 rpm for 48 h. DNA fractions were centrifuged again in separate gradients to improve the purity of the mtDNA (Sreenivasaprasad et al., 1992).

DNA digestion, Southern blotting and hybridization

Genomic DNA (1-3 μg) was digested for 16 h at 37°C with chosen hexameric restriction endonucleases (Promega, U.S.A.) according to manufacturer's instructions. Digested DNA was electrophoresed at 60 V on 0.8% w/v agarose gels using 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM-EDTA) containing 0.4 μg ml⁻¹ ethidium bromide (Hind III digested lambda-DNA and digested pGEM (Promega) were used as molecular size markers) and transferred to nylon membranes (Hybond N, Amersham, U.K.) (Sambrook, Fritsch & Maniatis, 1989). The clone pMY60, containing the complete 9 kb ribosomal DNA (rDNA) repeat unit from Saccharomyces carlsbergensis Hansen (Verbeet et al., 1983) was used as the probe for analysis of rDNA in BamH I, Cla I, EcoR I and Sma I genomic digests. For mitochondrial genome analysis, the mtDNA obtained from C. kahawae isolate IMI 338734 and a C. gloeosporioides isolate (isolate 311; Sreenivasaprasad et al., 1992) were used to probe EcoR I, Hinc II and Hind III digests of genomic DNA. Hybridization conditions were as described by Sreenivasaprasad et al. (1992).

Amplification of DNA and solid phase sequencing

Random amplification of genomic DNA was undertaken by polymerase chain reaction (PCR) in 100 μl reactions as
Fig. 2. Restriction fragment patterns of genomic DNA of *Colletotrichum kahawae* (CK) and *C. gloeosporioides* (CG) isolates digested with (a) *Eco* I and (b) *Hind* III and probed with mtDNA from *C. gloeosporioides*. Molecular size markers refer to *Hind* III-digested lambda-DNA and digested pGEM.

![Restriction fragment patterns](image)

Fig. 3. Dendrogram showing the inter-relationship of isolates of *Colletotrichum kahawae* and *C. gloeosporioides.*

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described by Sreenivasaprasad et al. (1992), using the 10 base primers A3 (AGTCAGCGAC), A11 (CAATCGCCGT), A13 (CAGCACCCAC), B6 (TGCTCTGCAC), B7 (GTTGACGCAG) and B10 (CTGCTGGGAC) (Operon Technologies Inc., U.S.A.) (Williams et al., 1990). PCR products (15 µl) were visualized on 1-4% w/v agarose gels containing ethidium bromide (0-4 µg ml⁻¹).

PCR amplification of the ITS 1 region of rDNA was carried out as described by Sreenivasaprasad et al. (1992), using the biotinylated ITS 1 and ITS 2 primers (White et al., 1990) supplied by Operon Technologies Inc. Direct solid phase sequencing of the PCR amplified ITS 1 region (Hultman et al., 1989) was done using Dynabeads (Dynal, Norway) according to the manufacturer's instructions. Biotin end-labelled DNA from a sample immobilized using 100 µl of prewashed beads, was suspended in 7 µl of sterile distilled water and used for sequencing by the chain-termination method (Sanger, Mickleu & Coulson, 1977). Non-biotinylated primers were used as sequencing primers with the T7 DNA polymerase sequencing kit (Promega). Sequencing reactions and electrophoresis were carried out as described by Gyllensten & Erlich (1988).

**Data analysis**

To produce a dendrogram, mtDNA restriction (*Eco* I) fragment profiles of all the isolates were subjected to cluster analysis by the Unweighted Pair Group Method with Averaging using the pattern matching software incorporated in the automated microbiology identification system (AMBIS) (Sreenivasaprasad, Mills & Brown, 1993).

The sequence data were aligned and analysed for distance matrix values using CLUSTAL V package (Higgins, Bleasby & Fuchs, 1992).
RESULTS

Restriction fragment patterns of rDNA

Ribosomal DNA restriction fragment patterns of *BamH*I and *Sma*I digested genomic DNA (Fig. 1a, b) indicated the homogeneity of the *C. kahawae* isolates. Restriction analysis with *Cla*I and *EcoR*I (data not presented) confirmed this observation. The size of the *C. kahawae* rDNA repeat unit was estimated, by summation of the sizes of the restriction fragments detected using these four restriction enzymes, to be between 8·4 and 9·18 kb. Isolates IMI 325946 (restriction pattern not shown) and IMI 325945, which were identical, and isolates of *C. gloeosporioides* (e.g. isolate CGcofA) from ripe coffee berries in Sri Lanka had different patterns to those from *C. kahawae* with each of the four restriction enzymes (Fig. 1a, b, data for *Cla*I and *EcoR*I not presented). The size of the rDNA repeat unit in these *C. gloeosporioides* isolates from coffee ranged from 9·3 to 10·72 kb.

Two isolates of *C. gloeosporioides* from avocado, one from Australia (JIA1) and one from New Zealand (918) and an isolate from mango from Sri Lanka (CGcofA) were included in this study as a comparison with CBD isolates and *C. gloeosporioides* from coffee berries. The CBD isolates had an identical rDNA pattern to isolate HAl when digested with *BamH*I (Fig. 1a) and had two bands in common with the three fruit isolates when digested with *Sma*I (2·0 and 0·98 kb; Fig. 1b) and *Cla*I (1·9 and 1·2 kb). The estimated molecular size of the rDNA repeat unit of the fruit isolates ranged from 8·4 to 10·18 kb.

Restriction fragment patterns of mtDNA

Restriction fragment patterns of genomic DNA from *C. kahawae* isolates digested with *EcoR*I and probed with *C. gloeosporioides* (Fig. 2a) or *C. kahawae* (data not shown) mtDNA were identical, as were patterns from *Hind*III digests.
(Fig. 2b) and Hinc II digests (data not presented). The estimated molecular size of the mtDNA from C. kahawae was 60 kb, based on the sum of the fragment sizes.

Many polymorphisms were observed when mtDNA patterns of C. kahawae and both coffee and fruit isolates of C. gloeosporioides were compared; an exception being the avocado isolate 918 from New Zealand (mtDNA molecular size of 57 kb) which had many bands in common with C. kahawae (Fig. 2a, b). The dendrogram produced using the mtDNA profiles (Fig. 3) showed that C. kahawae is more closely related to C. gloeosporioides avocado isolates 918 and 1072 from New Zealand (>96% similarity) than to C. gloeosporioides isolates from coffee (approx. 69% similarity to Sri Lankan isolate CGcofA and 82% similarity to isolate IMI 325945).

**RAPD analysis to detect genetic variability**

The possibility of genetic diversity among isolates of C. kahawae was tested using random 10 base primers. DNA from all 12 test isolates of C. kahawae, however, gave rise to identical RAPD patterns with each of the six primers used, for example, with primer A3 major fragments of 1.95, 0.64, 0.52 and 0.35 kb were amplified (Fig. 4). The avocado C. gloeosporioides isolate 918, with primer A3, shared the first three of these fragments while no common fragments occurred among the other C. gloeosporioides isolates included in the study (Fig. 4).

**Sequencing of the ITS 1 region of C. kahawae and C. gloeosporioides**

The ITS 1 region of C. kahawae and C. gloeosporioides isolates obtained from coffee was shown to be 171 bases. No variation occurred in the nucleotide sequence of the C. kahawae isolates (Fig. 5), i.e. they showed 100% sequence homology (Table 2). The ITS 1 region of C. kahawae showed 98.2% homology with the two C. gloeosporioides isolates obtained from coffee in Africa and two from coffee in Sri Lanka. A third C. gloeosporioides isolate from coffee in Sri Lanka showed 98.8% homology with C. kahawae (Fig. 5, Table 2).

**DISCUSSION**

Results of rDNA and mtDNA RFLP analyses and RAPD analysis indicate that isolates of the CBD pathogen represent a genetically homogeneous population with a common origin, which has subsequently become distributed through several countries of Africa. Although a discrete group, there are, however, very close similarities in the rDNA and mtDNA between the CBD pathogen and avocado isolates of C. gloeosporioides obtained from New Zealand and Australia. This suggests that the CBD pathogen could have arisen, on a natural progenitor of Coffea arabica L. (Robinson, 1974) or on C. arabica itself in Africa, from a C. gloeosporioides isolate with close genetic homogeneity to these avocado isolates. We are not, however, suggesting that the genetic similarity observed is, in any way, related to the pathogenicity of these isolates. Considering the variation within C. gloeosporioides, the host origin of the progenitor of C. kahawae in Africa could not be predicted from data available. Alternatively, the CBD pathogen may have arisen as a result of chromosomal rearrangement during somatic growth or parasexual recombination, as has been suggested for isolates of C. gloeosporioides with preference for other crop hosts (Masel et al., 1990). In asexual populations of C. gloeosporioides, heterokaryosis is likely to be of major significance in the rapid adaptation of a pathogen to a new host. The clonal nature of the isolates of C. kahawae could suggest that the success of the CBD pathogen on cultivated C. arabica in Africa has resulted in little further change in this organism, although some apparent differences in aggressiveness and morphology have been observed between isolates from Angola and Malawi and those from Kenya (Rodrigues et al., 1991).

C. gloeosporioides populations on coffee, in areas where the crop is extensively grown, are variable but none shows the cultural or pathogenic characteristics of the CBD pathogen (Waller, 1982). In the present study, rDNA and mtDNA analyses and RAPD analysis enabled the CBD pathogen to be recognized as a distinct group within C. gloeosporioides. The degree of diversity between the CBD pathogen and isolates of C. gloeosporioides from coffee is, however, no greater than the variation observed among more than 100 isolates of C. gloeosporioides from various crop hosts of world-wide origin (e.g. Hodson et al., 1992). In addition, the genetic homogeneity of the CBD pathogen is not unique. Isolates of C. gloeosporioides from mango obtained from world-wide sources appeared genetically uniform and other C. gloeosporioides isolates could be genetically grouped according to their host source within geographic localities (Braithwaite et al., 1990; Hodson et al., 1992; Mills et al., 1992b). However, isolates of C. gloeosporioides obtained from different hosts rarely had the same rDNA or mtDNA restriction banding patterns (Hodson et al., 1992) or

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**Table 2. Distance matrix and percent homology between the ITS 1 sequence of C. kahawae and C. gloeosporioides isolates**

<table>
<thead>
<tr>
<th></th>
<th>C. kahawae</th>
<th>IMI 325945</th>
<th>IMI 325946</th>
<th>CGcofA</th>
<th>CGcofC</th>
<th>CGcofR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. kahawae*</td>
<td>—</td>
<td>0.018</td>
<td>0.018</td>
<td>0.018</td>
<td>0.012</td>
<td>0.018</td>
</tr>
<tr>
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<td>—</td>
<td>0</td>
<td>0.018</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IMI 325946</td>
<td>98.2</td>
<td>100</td>
<td>—</td>
<td>0</td>
<td>0.018</td>
<td>0</td>
</tr>
<tr>
<td>CGcofA</td>
<td>98.2</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>0.018</td>
<td>0</td>
</tr>
<tr>
<td>CGcofC</td>
<td>98.2</td>
<td>98.2</td>
<td>98.2</td>
<td>98.2</td>
<td>—</td>
<td>0.018</td>
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<tr>
<td>CGcofR</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>98.2</td>
<td>—</td>
</tr>
</tbody>
</table>

* C. kahawae = isolates IMI 229393, IMI 190857, IMI 319406, IMI 319418, IMI 348838, IMI 301220, IMI 338734, IMI 311655, IMI 338730, IMI 300964.

For details of isolates, see Table 1

Distance matrix is given above diagonal; percent homology is given below diagonal (calculated using CLUSTAL v package, Higgins et al., 1992).
RAPD banding patterns (Mills et al., 1992b) indicating extensive genotypic variation within *C. gloeosporioides*.

One of the most appropriate means for determining relationships among fungi is to analyse the non-conserved regions of the rDNA genes (Förster et al., 1990; Illingworth et al., 1991). Sequencing of the variable ITS I region of the rDNA repeat unit has shown divergence of up to 5% among isolates of *C. gloeosporioides* from different fruit hosts, with divergence between *C. gloeosporioides* isolates and the apparently most closely related species *C. musae* (Berk. & Curt.) von Arx being greater than 8% (Mills et al., 1992a). Divergence between *C. gloeosporioides* and the strawberry anthracnose pathogen *C. fragariae* Brooks has been calculated at 3-5% (Sreenivasaprasad et al., 1992) and it has been suggested that *C. fragariae* is not a species distinct from *C. gloeosporioides* (von Arx, 1957), although this is a matter of dispute (Smith & Black, 1990). The largest interspecific divergence between CBD isolates and isolates of *C. gloeosporioides* from other fruit crops was < 3% (cf. Fig. 5, and Mills et al., 1992a). When comparing this divergence value with the 5% value found within fruit isolate groups it is apparent that the CBD pathogen, although described as a distinct species (Sutton, 1992; Waller et al., 1992), falls within the group species concept of *C. gloeosporioides*. It may be more appropriate to differentiate CBD isolates at a sub-specific level, e.g. as *C. gloeosporioides* var. *kahatae*, however, such subspeciation of *C. gloeosporioides* would require thorough assessment of infraspecific divergence.

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**REFERENCE**


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