African Journal of Biotechnology Vol. 3 (3), pp. 195-198, March 2004
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2004 Academic Journals

Full Length Research paper

Colletotrichum circinans and *Colletotrichum coccodes* can be distinguished by DGGE analysis of PCR-amplified 18S rDNA fragments

Olajire Fagbola[1+] and Mathew M. Abang[2]*

[1]Federal Biological Research Centre for Agriculture (BBA), Messeweg 11/12, D-38104 Braunschweig, Germany.
[2]German Collection of Microorganisms and Cell Cultures (DSMZ), Messeweg 11/12, D-38104 Braunschweig, Germany.

Accepted 29 December 2003

The rDNA 18S region of *Colletotrichum circinans* and *C. coccodes* was amplified by PCR to evaluate this DNA region as a tool for species delineation. PCR amplification of the 18S of both species produced 1.65 Kb long fragments that covered most of the entire 18S rDNA molecule. DGGE analysis of the amplified fragments distinguished *C. circinans* from *C. coccodes* isolates. This result provides molecular evidence that supports the current treatment of *C. circinans* as a species distinct from *C. coccodes*, in spite of the failure of previous attempts at genetic differentiation of the two species based on RFLP analysis of the rDNA ITS region.

Key words: DGGE, *Colletotrichum circinans*, *Colletotrichum coccodes*, molecular differentiation, species delineation.

INTRODUCTION

The revision of *Colletotrichum* by von Arx (1957) was a landmark in the taxonomy of plant pathogens, in which 750 "species" of *Colletotrichum* were reduced to 11 taxa based on morphology rather than host-specificity. The number of "accepted" species has now increased to 39, based on more detailed studies of morphology, cultural characteristics, and pathogenicity (Sutton, 1980, 1992). Several taxa considered as host specialized forms by von Arx (1957) were recognized as full species by Sutton (1992). For instance, Sutton (1992) regarded *C. circinans* as distinct from *C. coccodes* and other *Colletotrichum* species, while von Arx (1957) regarded *C. circinans* as an *Allium*-specific form of *C. dematium* (*C. dematium* f.sp. *circinans*). The complete host range of most *Colletotrichum* species is as yet unknown, and Sutton's treatment of the genus has been criticized for being based on the uncritical assumption that *Colletotrichum* species are host-specific (Cannon et al., 2000). Conidia of *C. coccodes* are described as 16-24 μm long by 3-4 μm wide, straight, fusiform and abruptly tapered while those of *C. circinans* are 19-21 x 3.5 μm, falcate, fusiform and gradually tapered to each end (Sutton, 1992). However, many *Colletotrichum* species produce secondary conidia in culture that are generally smaller and more varied in shape, especially when the culture is old (Cannon et al., 2000). The common occurrence of intermediate morphological features, and the recognition that the currently "accepted" species may actually be host-specific forms of species complexes or aggregates has led to the use of biochemical and molecular markers to verify species boundaries within *Colletotrichum* (Cannon et al., 2000; Abang et al., 2002).

Analysis of the ITS regions, as well as the D1 and D2 domains of the nuclear rDNA, have been used extensively to resolve taxonomic questions within *Colletotrichum* (Sreenivasaprasad et al., 1996, Freeman et al., 2000). Martín and García-Figuerez (1999) and Abang et al. (2002) failed to distinguish *C. coccodes* from

*Corresponding author. Present address: IRAD, P. O. Box 2123 Messa-Yaoundé, Cameroon, and Germplasm Program, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 5466, Aleppo, Syria. E-mail: m.abang@cgiar.org.
+Present address: Dept of Agronomy, University of Ibadan, Ibadan, Nigeria.

Abbreviation: ITS, internal transcribed spacer; DGGE, denaturing gradient gel electrophoresis.

C. circinans based on restriction analysis of their ITS sequence. They hypothesized that the two species are identical and could belong to the same taxon, in spite of apparent morphological differences. DGGE analysis of PCR-amplified rDNA has been used for species delineation in fungal genera such as *Pleomassaria* and *Melampsoridium* (Paavolainen *et al*. 2000), and DGGE analysis of 18S rDNA has been successfully applied in molecular differentiation of *C. gloeosporoides* and *C. acutatum* (Fagbola et al., 2001). In this study, we applied the technique to determine if molecular evidence supports the treatment of *C. circinans* as a species distinct from *C. coccodes*.

RESULTS AND DISCUSSION

Total genomic DNA was extracted from isolates of *C. coccodes* (DSM 62126, 66376, 70882, 70879, DSM 2492, 70880) and *C. circinans* (67846 and 71247) using a modified CTAB procedure (Abang et al., 2002). The oligonucleotide primers, FR1 (fungus-specific reverse primer 1) and NS1 (forward primer), of Vainio and Hantula (2000) were used for PCR amplification of the small subunit rDNA of the isolates (Gomes et al., 2003). The primer pair FR1 + NS1 produced 1.65 Kb long PCR-fragments that covered most of the entire SSU rDNA molecule. Good resolution of all of the products was achieved using the denaturing gradients of 18-43 % and no artefacts bands were observed (Figure 1).

The *Colletotrichum* strains were separated into two distinct groups based on the migration rate of their amplified 18S fragments following DGGE (Figure 2). The amplified 18S bands of *C. circinans* migrated faster (band "b") and were clearly distinct from those of *C. coccodes*. *Colletotrichum coccodes* isolates could be easily distinguished by the slow migration rate of their amplified 18S bands in denaturing gradient gels (band "a"). The migration of amplified 18S rDNA samples within each *Colletotrichum* species was similar, suggesting that there was little intraspecific variation. It is also likely that intraspecific variation present was not properly resolved by DGGE. However, this was not tested by sequencing.

DGGE is an electrophoretic technique that efficiently separates DNA molecules according to their size, as well as sequence differences (Myers et al., 1986, Lessa, 1992). In comparison to DNA sequencing, DGGE is a simple and relatively cheap method that can be used in genetic fingerprinting assays of large numbers of samples. Application of this technique to a 1.65 Kb SSU rDNA fragment was previously shown to be an efficient method for differentiating taxa within the *C. gloeosporioides* group species associated with anthracnose disease of yam in Nigeria (Fagbola et al, 2001). Also, DGGE analysis of PCR-amplified SSU rDNA clearly differentiated *C. gloeosporioides* from *C. acutatum*. DGGE of PCR-amplified 18S fragments

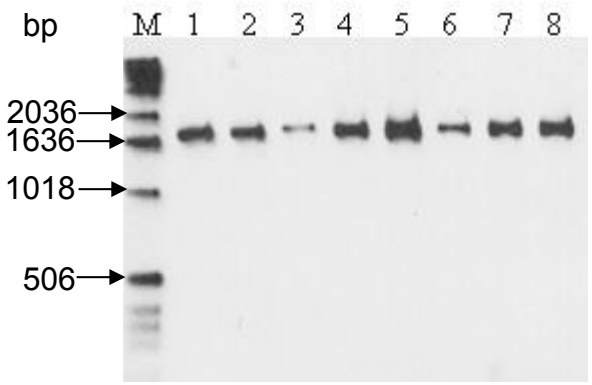

Figure 1. Electrophoretic pattern of PCR-amplified 18S ribosomal DNA on ethidium bromide-stained agarose gel, depicting a single 1.65 Kb long fragment for all the *Colletotrichum* strains analysed in this study. M = molecular size marker (1 Kb DNA ladder, Gibco BRL). Total genomic DNA was extracted from aerial mycelium/conidia scraped off 7-day-old cultures using a modified CTAB procedure (Abang et al., 2002). DNA quality was visually assessed on 1% agarose gel following electrophoresis, and the concentration was measured using a Dyna Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., USA). The oligonucleotide primers, FR1 (fungus-specific reverse primer 1) and NS1 (forward primer), of Vainio and Hantula (2000) that target the 18S subunit of the fungal genome were used for PCR amplification. PCR was performed with a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CT, USA). PCR amplification of DNA was carried out in 25 µl reaction mixtures containing 2.5 µl of 10X reaction buffer, 3.5 µl of 25 mM $MgCl_2$, 2.5 µl 2.0 mM dNTP mixtures, 0.5 µl DMSO (2% v/v), 0.5 µl each of the primers at 10pmol concentration, 0.25 µl of Stoffel fragment, 1.0µl template DNA, and 13.75µl water. Amplification condition was 8 min at 94^{o}C, followed by 25 cycles of 30s at 94^{o}C, 45s at 48^{o}C, 3 min at 72^{o}C, and final extension for 10 min at 72^{o}C. Products were first analysed by electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining.

provided clearly interpretable results suggesting that this method could be useful in further verification of species delineation among the 39 "accepted" species currently defined within *Colletotrichum* based on cultural characteristics (Sutton, 1992). In previous studies, restriction analysis did not reveal any difference in the ITS sequence of *C. coccodes* and *C. circinans* (Martin and Garcia-Figuerez, 1999; Abang, 2003). However, a more intensive usage of different enzymes might have differentiated the two species. It may seem surprising that differences not evident in the variable ITS region were revealed in the conserved 18S gene; however, Cannon et al. (2000) have noted that the distinction between the ITS and 18S as variable and conserved regions, respectively, is not absolute. In fact, functional ribosomal sequences have been used for species and intraspecific group definition in *Colletotrichum* (Sheriff et al., 1994; Johnston and Jones, 1997).

This is the second report of the application of DGGE of PCR-amplified 18S rDNA for genetic differentiation of *Colletotrichum* species, after the differentiation of *C.*

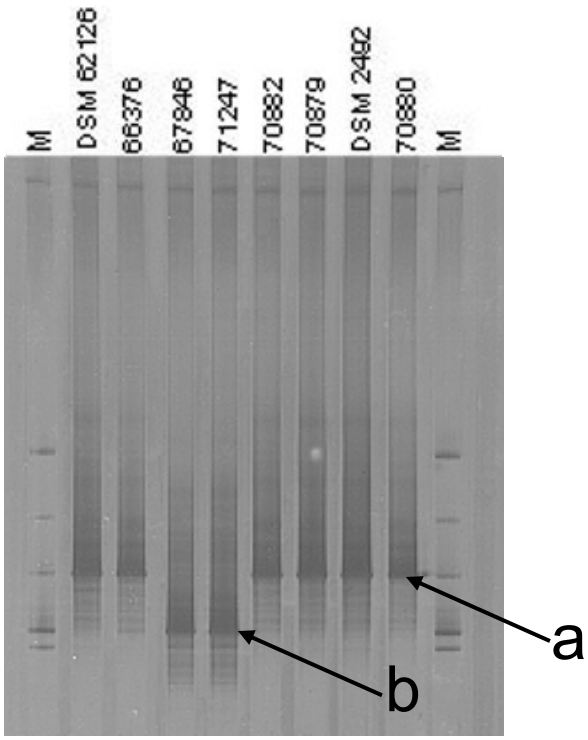

Figure 2. Denaturing gradient gel electrophoresis profiles depicting differential migration of PCR-amplified 18S ribosomal DNA fragments of *C. coccodes* isolates DSM 62126, 66376, 70882, 70879, DSM 2492, 70880 (arrow points to slow-migrating bands "a"), and *C. circinans* isolates 67846 and 71247 (arrow points to fast-migrating bands "b"). The following fungal species, from top to bottom, were used as standards (Lanes M): *Colletotrichum* sp., *Sclerotium tuliparum*, *Trichoderma harzianum*, *Myrothecium cinctum*, *Ustilago nuda*, *Myrothecium leucotrichum*, and *Penicillium simplicissimum*. DSM isolates were kindly supplied by Dr Peter Hoffmann from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), while Dr Helgard Nirenberg provided the other *Colletotrichum* strains from the fungal collection of the Federal Biological Research Centre for Agriculture (BBA, Berlin, Germany). PCR products from all isolates were analysed using DGGE (D GENE System; Bio-Rad, Inc., Hercules, CA) (Gomes et al., 2003). The DGGE solution was made of 7.5% (w/v) acrylamide/bisacrylamide (37.5:1) gels. The denaturant gradient was 18-43% which were produced with 100% denaturing solution containing 40% deionized formamide and 7 M urea. The gels were run in 1X TAE-buffer at a constant temperature of 58ºC and 180V for 18 h. The gels were silver stained to visualise the electrophoretic mobility pattern of the isolates. Silver staining solutions used were 10% (v/v) ethanol plus 0.5% acetic acid for fixation, freshly made 0.1% (w/v) silver nitrate for staining, freshly prepared developing solution containing 0.01% (w/v) sodium borohydride, 0.15% formaldehyde, 1.5% (w/v) NaOH and 0.75% (w/v) sodium carbonate solution to stop the development. Gels were mummified and dried for documentation and analysis.

gloeosporioides and *C. acutatum* (Fagbola et al., 2001). The technique may be applied in any of the many instances where multiple *Colletotrichum* species cause anthracnose disease on the same host (e.g. Martín and García-Figueres, 1999). Because electrophoretic behaviour directly reflects differences in nucleotide composition of the fragments screened, the assay provides clear evidence of polymorphism among the taxonomic entities investigated. This study was handicapped by our inability to find more *C. circinans and C. coccodes* isolates for analysis. It would be necessary to analyse more strains within each species, and to obtain DNA sequence data from multiple loci (including the SSU rDNA gene) to establish the taxonomic boundaries around *C. circinans and C. coccodes*.

Results obtained in the present study support the treatment of *C. circinans* as a species distinct from *C. coccodes*, in contrast to previous studies (Martín and García-Figuerez, 1999; Abang et al., 2002) that failed to find molecular evidence in support of Sutton's (1992) treatment of the two taxa as distinct species. Clarification of species boundaries in *Colletotrichum* is a matter of urgency and great practical importance, especially for plant pathologists. Not only does our ability to accurately identify disease causal agents depend on it but precise data on host ranges and geographic distributions of species cannot be assembled until these problems are resolved.

ACKNOWLEDGEMENTS

The first author was supported by AvH Stiftung, Germany. The authors would like to thank Drs Kornelia Smalla (BBA, Braunschweig) and Stephan Winter (DSMZ, Braunschweig) for their support of this work, and Drs Peter Hoffmann (DSMZ, Braunschweig, Germany) and Helgard Nirenberg (BBA, Berlin, Germany) for kindly providing *C. coccodes* and *C. circinans* reference isolates.

REFERENCES

Abang MM (2003) Genetic diversity of *Colletotrichum gloeosporioides* Penz. causing anthracnose disease of yam (*Dioscorea* spp.) in Nigeria. Bibliotheca Mycologica Vol 197. J. Cramer Science Publishers, Berlin, Stuttgart.

Abang MM, Winter S, Green KR, Hoffmann P, Mignouna HD, Wolf GA (2002) Molecular identification of *Colletotrichum gloeosporioides* causing anthracnose of yam in Nigeria. Plant Pathol. 51:63-71.

von Arx JA (1957). Die Arten der Gattung *Colletotrichum* Cda. Phytopathol. Zeitschrift 29:413-468.

Cannon PF, Bridge PD, Monte E (2000) Linking the past, present, and future of *Colletotrichum* systematics. In: Prusky D, Freeman S, Dickman MB (eds) *Colletotrichum*: host specificity, pathology, and host-pathogen interaction: APS Press, Minnesota, USA, pp 1-20

Fagbola O, Abang MM, Smalla K, Winter S (2001). Molecular fingerprinting with DGGE and rep-PCR resolves biotype diversity and reveals genetic relationships within *Colletotrichum gloeosporioides* from yam. [Abstract PFDO1] *BIOspektrum* – Sonderausgabe zur VAAM Jahrestagung 2001:89.

Freeman S, Minz D, Jurkevitch E, Maymon M, Shabi E (2000). Molecular analyses of *Colletotrichum* species from almond and other fruits. Phytopathol. 90:608-614.

Gomes NCM, Fagbola O, Costa R, Rumjanek NG, Buchner A, Mendona-Hagler L, Smalla K (2003). Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. Appl.

Environ. Microbiol. 69:3758–3766.
Johnston PR, Jones D (1997). Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia 89: 420-430.
Lessa EP (1992). Rapid surveying of DNA sequence variation in natural populations. Mol. Biol. Evol. 9: 323-330.
Martín MP, García-Figueres F (1999). *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* cause anthracnose on olives. Eur. J. Plant Pathol. 105:733-741.
Myers RM, Maniatis T, Lerman LS (1986). Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods in Enzymology 155: 501-527.
Paavolainen L, Kurkela T, Hallaksela A, Hantula J (2000). *Pleomassaria* sipari is composed of two biological species, both of which occur in dead twigs of *Betula pendula* and *B. pubescens*. Mycologia 92: 253-258.
Sherriff C, Whelan MJ, Arnold GM, Lafay J-F, Brygoo Y, Bailey JA (1994). Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Exp. Mycol. 18: 121-138.
Sreenivasaprasad S, Mills PR, Meehan BM, Brown AE (1996). Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome 38:499-512.
Sutton BC (1980). The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervula and Stromata. Commonwealth Mycological Institute, Kew, Surrey, England.
Sutton BC (1992). The genus *Glomerella* and its anamorph *Colletotrichum*. In: Bailey JA, Jeger MJ, (eds) *Colletotrichum*: Biology, Pathology and Control: CAB International, Wallingford, England, pp 1-26.
Vainio EJ, Hantula J (2000). Direct analysis of wood-inhabiting fungi using denaturing gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104: 927-936.