A COMPARISON OF *COLLETOTRICHUM* SPECIES ASSOCIATED WITH BERRY DISEASES OF *COFFEA ARABICA* L.

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ABSTRACT
Fourty isolates of Colletotrichum species associated with coffee berry anthracnose in PNG have been characterised and identified on the basis of cultural, morphological and molecular characteristics. Twenty nine isolates were identified as C. gloeosporioides while the remaining 11 were identifiesd as C. acutatum. None of the isolates resembled characteristics common to C. kahawae.

1.0 INTRODUCTION

Fungi of the genus *Colletotrichum* include some 900 species (Sutton, 1992) and are most commonly associated with anthracnose symptoms in the respective host plant tissues. Despite the large number of species only 3, *C. kahawae* J. M. Waller & P.D. Bridge, sp. nov (formerly *C. coffeanum* Noack), *C. gloeosporioides* Penz, and *C. acutatum* Simmonds, have been isolated from coffee (Hindorf, 1970). *C. kahawae* attacks all stages of the crop from flowering to ripe berries and can result in yield loses as high as 80% (Griffiths et al., 1971) including the abandonment of coffee growing as has happened in some parts of Africa (Turner, 1992). This compares with *C. gloeosporioides* where the attack is restricted to the ripe berries only and yield losses as high as 40% (Mignucci, et al., 1985). *C. acutatum* is also associated with ripe berry anthracnose but evidence is lacking in the nature of its pathological or saprophytic association with berry anthracnose and resultant yield loss.

C. kahawae is a very serious threat to economic coffee production in all countries where the fungus has not been reported including PNG. Therefore a general knowledge on the features characteristic of *C. kahawae* is useful in diagnosing the cause of berry diseases in PNG. The purpose of this paper is to present data obtained on *Colletotrichum* species isolated from coffee berry anthracnose in PNG and compare it with *C. kahawae* causing coffee berry disease (CBD) in African countries.

2.0 MATERIALS AND METHODS

Samples of diseased berries were collected from various localities in 5 provinces (Southern Highlands, Wabag, Western Highlands, Chimbu and Eastern Highlands) during the months of April, May, June and July, 2002. A total of 40 sites were visited for sample collection and for each site a pure culture of single spore isolate was obtained which was used in the cultural, morphological and molecular studies. The isolates were code named based on provincial, electorate and sampling site codes (Table 1).

Table 1 Isolats derived from provincial, electoral and site codes

Isolate Code	Province	Provincial Code	Electorate	Electoral Code	Sampling Site	Site Code
1001a	Southern	1	Mendi	001	Mendi1	a
1001b	Highlands				Mendi2	b
1001c	(SHP)				Mendi3	c
1001d					Mendi4	d
1002a			Imbonggu	002	Kaugel1	a
1002b					Kaugel2	b
2007a	Enga	2	Wabag	007	Wabag1	a
2007b					Wabag2	b
2008a			Wapenamenda	008	Wapenamenda1	a
2008b					Wapenamenda2	b
2008c					Pausa	c
3012a	Western	3	North Wahgi	012	Numans	a
3012b	Highlands				Banz	b
3013a	(WHP)		South Wahgi/	013	Panga	a
3013b			Angalimp		Kudjip	b
3013c					Minj	c
3014a			Tambul/	014	Togoba1	a
3014b			Nebilyer		Togoba2	b
3014c					Togoba3	c
3016a			Hagen	016	Keltiga1	a
3016b					Keltiga2	b
3016c					Dobel	c
3016d					Mt. Ambra	d
3018a			Dei	018	Nunga	a
3018b					Kinjibi	b
4019a	Chimbu	4	Chuave	019	Chuave	a
4022a			Kerowagi	022	Kunabau	a
4023a			Kundiawa/	023	Mindima	a
4023b			Gembol		Wandi	b
4023c					Kundiawa	c
4024a			Sinasina/ Yonggamugl	024	Masual	a
5025a	Eastern	5	Daulo	025	Watabung	a
5025b	Highlands				Asaro	b
5026a	(EHP)		Goroka	026	Kabiufa	a
5026b					Kamaliki	b
5027a			Henganofi	027	Kompri	a
5028a			Kainantu	028	Yonki	a
5030a			Obura/	030	Aiyura	a
5030b			Wonenara		Kovuta	b
5030c					Urara	c

Cultural characteristics were studied on potato dextrose agar (PDA) amended with 0.02% streptomycin sulphate. Spore morphology was described from specimen fixed in lactophenol cotton blue. Each isolate was identified using Sutton's (1980) identification keys.

Molecular techniques involving the polymerase chain reaction (PCR) technology was used as an additional tool for isolate identification. The methodology used follows that of Manaut et al. (2001) with some modifications. Mycelium for DNA extraction was collected directly from cultures grown on PDA. Amplification of the internal transcribed spacer (ITS) regions between 18S and 28S including the 5.8S segment of the ribosomal deoxyribonucleic acid (rDNA) was carried out using the universal primers ITS1 and ITS4. The restriction enzymes *DpnII*, *HhaI*, *HinfI*, *TaqI* and *HpaII* were used for the analysis of restriction fragment length polymorphism (RFLP) of the ITS region.

In order to determine the rDNA sequence of the ITS region the following procedure was followed. The PCR product of the ITS region was purified with Nucleospin Extract Kit and ligated into the pGEM-T easy vector following the manufacture's protocols. The ligation product was transferred into *Escherichia coli* compotent cells strain 109. DNA plasmid was prepared from the transformed *E. coli* cells using Ultra mini plasmid preparation kit following the manufacture's protocol. Sequencing reactions were primed on both strands of plasmid DNA using the SP6 and T7 promoter sequences. Sequencing of the plasmid DNA was done by the Australian Genome Research Facility at the University of Queensland. The sequence was manually aligned and blast searched on the database to determine sequence homology with already sequenced ITS region of rDNA of *Colletotrichum* species.

2.5 RESULTS

The cultural and morphological features have been described for all the 40 isolates of *Colletotrichum* with only the features relevant for species identification summarised under the categories of a) whole colony, b) mycelium and c) reproductive structures (Table 2). Species identification given in table 2 is based on Sutton (1980).

Differences as well as similarities are evident among the 40 isolates in one or more of the features used to characterise each isolate. For example variation in the colony growth rate ranged from 4.0 - 11.8 mm d^{-1} with 11 isolates (1001a, 1001b, 1001d, 1002b, 2007b, 2008a, 2008b, 3013a, 3016a, 3016c, and 3016d) characterised by relatively slower growth rates (<5 mm d^{-1}) compared to the rest of the isolates. Differences between the isolates in some of the other characteristics included colony colour (wool white to dirty white/grey), mycelial form (loose, compact) and elevation (low, moderate and high) and, presence of sclerotial bodies. However, these differences were not as consistent as the growth rates within the slow and fast growing groups of isolates.

The PCR product of the entire ITS region between 18S and 28S of the rDNA showed identical banding pattern for all the 40 isolates with the size of the amplified products

Table 2 **Descriptions of some features of the colony, mycelium and reproductive structures of 40 isolates of *Colletotrichum* from PNG and species identification**

Isolate	Descriptive features										Species Identification[k]
	Whole Colony			Mycelium			Reproductive structures				
	Colony			Mycelial		Sclerotial	Acervuli[g]	Setae[h]	Conidium		
	colour[a]	growth[b]	margin[c]	form[d]	elevation[e]	bodies[f]			size[I]	shape[j]	
1001a	1	4.4	2	1	2	0	1	0	11.4x3.3	1	a
1001b	2	4.6	1	2	1	0	1	0	15.1x2.3	1	a
1001c	1	11.6	2	1	1	1	2	1	16.2x4.2	2	g
1001d	3	4.6	2	1	2	0	1	0	13.2x2.8	1	a
1002a	3	10.7	2	1	2	1	2	1	14.6x4.2	2	g
1002b	3	4.6	2	1	2	0	1	0	14.0x2.1	1	a
2007a	1	9.8	2	1	1	1	2	1	15.9x5.4	2	g
2007b	3	4.5	2	1	1	0	1	0	11.6x3.5	1	a
2008a	2	4.1	2	1	2	0	1	0	15.0x4.2	1	a
2008b	1	4.3	1	1	1	0	1	0	14.4x2.4	1	a
2008c	1	11.2	2	1	1	0	2	1	15.2x3.9	2	g
3012a	5	10.0	1	1	3	1	2	1	19.5x4.6	3	g
3012b	5	9.9	1	1	3	1	2	1	16.4x5.4	2	g
3013a	1	4.0	2	1	2	0	2	1	15.9x4.9	2	a
3013b	1	10.9	1	1	2	0	2	1	16.0x4.8	2	g
3013c	4	11.1	1	2	1	0	0	1	14.8x5.2	2	g
3014a	3	10.4	1	1	3	1	2	1	14.7x4.7	2	g
3014b	3	11.4	1	1	3	1	2	1	14.6x4.2	2	g
3014c	3	10.8	1	1	3	1	2	1	15.4x4.8	2	g
3016a	1	5.0	1	1	2	0	1	0	12.9x4.3	1	a
3016b	1	9.7	2	1	2	1	2	1	15.9x3.2	2	g
3016c	2	4.8	2	2	1	0	1	0	11.6x2.2	1	a
3016d	2	5.0	2	2	1	0	1	0	13.5x2.4	1	a
3018a	1	10.1	2	1	2	0	2	1	13.1x3.9	2	g
3018b	1	10.6	1	1	2	0	2	1	15.2x4.7	2	g
4019a	2	10.2	1	1	2	0	2	1	16.0x4.1	2	g
4022a	4	10.1	1	2	1	1	2	1	15.6x5.1	2	g
4023a	4	10.3	1	2	1	1	2	1	15.9x4.7	2	g
4023b	4	10.8	1	2	1	0	2	1	15.0x5.2	2	g
4023c	1	9.8	2	1	2	0	2	1	14.2x4.0	2	g
4024a	3	11.2	1	1	2	1	2	1	15.1x4.1	2	g
5025a	1	10.5	1	1	2	0	2	1	14.7x4.5	2	g
5025b	1	11.4	1	1	2	0	2	1	15.6x4.2	2	g
5026a	5	10.4	1	1	3	1	2	1	14.8x5.1	2	g
5026b	5	11.0	1	1	3	1	2	1	16.4x4.8	2	g
5027a	3	11.5	1	1	3	1	2	1	15.1x4.6	2	g
5028a	3	11.5	1	1	3	1	2	1	14.9x4.0	2	g
5030a	3	11.8	1	1	3	1	2	1	19.8x5.2	3	g
5030b	3	10.1	1	1	3	1	2	1	15.6x5.0	2	g
5030c	5	10.5	1	1	3	1	2	1	14.8x4.8	2	g

[a]Colony colour, 1 = white, 2 = grey, 3 = white to dirty white, 4 = dirty white, 5 = wool white
[b]Colony growth, average growth per day given in mm
[c]Colony margin, 1 = regular, 2 = irregular
[d]Mycelial form, 1 = loose, 2 = compact

[e]Mycelial elevation, 1 = low, 2 = moderate, 3 = high
[f]Sclerotial bodies, 0 = absent, 1 = present
[g]Acervuli, 0 = rare, 1 = poor, 2 = abundant
[h]Setae, 0 = absent, 1 = present
[i]Conidia size, conidia dimensions given in micrometers
[j]Conidia shape, 1 = cylindrical/fusiform, 2 = cylindrical/straight, 3 = cylindrical with one end tapering
[k]Species identification, a = *C. acutatum*, g = *C. gloeosporioides*

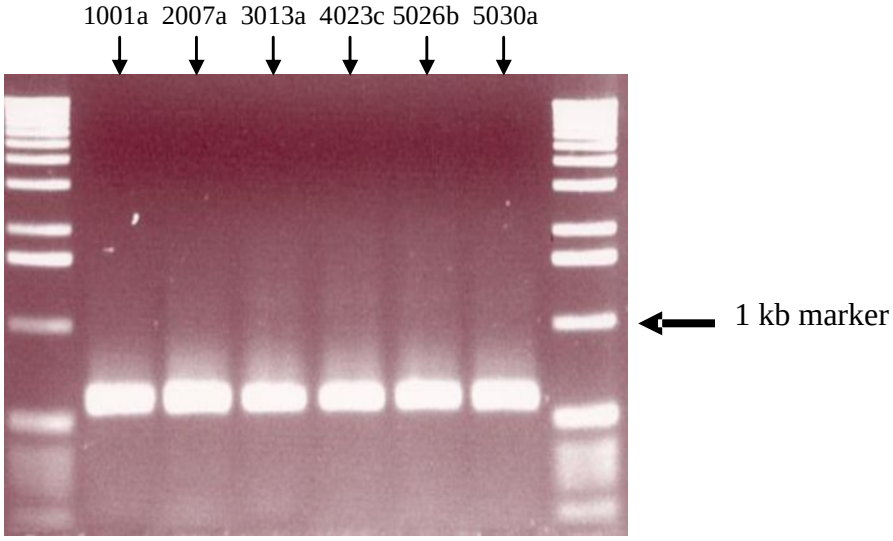

Figure 1 PCR amplification of ITS region of rDNA for *Colletotrichum* isolates 1001a, 2007a, 3013a, 4023c, 5026b and 5030a

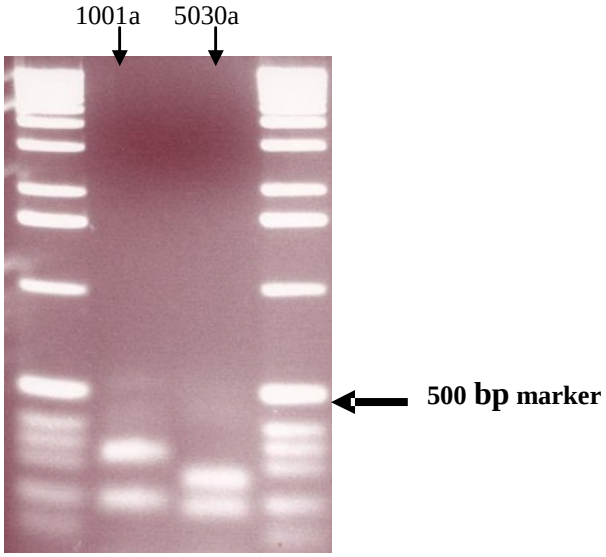

Figure 2 RFLP banding pattern for isolates 1001a and 5030a representing slow and fast growing isolates respectively

measuring just over 500 base pairs long as illustrated by the 6 isolates (figure 1). The 6 isolates represent 5 provinces and 3 forms of conidia shapes. All the restriction enzymes

tested were not able to separate the isolates into different RFLP groups except for restriction enzyme *DpnII*. Digestion with *DpnII* separated the isolates into 2 different RFLP groups as represented by isolates 1001a and 5030a (figure 2). All the 11 slow growing isolates fell under the RFLP group of 1001a while the remaining 29 isolates fell under 5030a. Although isolates 3012a and 5030b differed in conidial shape and size none of the restriction enzymes tested proved that these 2 isolates were genotypically different from the rest of the fast growing isolates. On the contrary they were grouped together with the rest of the fast growing isolates by enzyme *DpnII*. Indeed, the mean conidia size of these 2 isolates fall within the size range of some fast growing isolates such as 1001c and 2007a.

DNA sequence analysis of the two RFLP groups showed that the RFLP group represented by isolate 1001a was 584 base pairs long while those represented by 5030a was 574 base pairs long. By comparing the DNA sequence of the ITS regions of the two isolates with data on the database by using the BLAST (Altschul, et al., 1997) search program it was found that isolate 5030a was similar (e-value 0.0) to *C. gloeosporioides* while isolate 1001a was similar to *C. acutatum*. There was 99% DNA sequence homology between isolate 5030a and *C. gloeosporioides* accession reference gi/31745580/gb/AY245021.1. Differences in the DNA sequence were detected at nucleotide 427 and 496. Similarly the DNA sequence homology between isolate 1001a and *C. acutatum* accession reference gi/24459953/dbj/AB042301.1 was 99% with differences at nucleotide 129, 448 and 515.

2.6 DISCUSSION

The species identification of the 40 isolates of *Colletotrichum* from PNG was made possible by both molecular techniques and conventional methods relying on the cultural and morphological features. The cultural and morphological features described for 27 isolates fall within Sutton's (1980) species identification for *C. gloeosporioides*. The variation in cultural features observed in this group of isolates agrees with earlier studies (Hocking, 1966; Gibbs, 1969; Hindorf, 1970; Hindorf and Muthappa, 1974; Muthappa, 1974, Waller et al., 1993) and is characteristic of *C. gloeosporioides*.

Two isolates, 3012a and 5030b, had conidia sizes outside of the range observed by Hocking (1966), Hindorf (1970) and Hindorf and Muthappa (1974) for *C. gloeosporioides* isolated from coffee. It is not uncommon for *C. gloeosporioides* to produce conidia in the range observed in this study because the size range covered in Sutton (1980) is 9 - 24 x 3 - 4.5. Manaut et al. (2001) reported conidial dimensions ranging from 9.2-29.5 x 2.3-5.8 for *C. gloeosporioides* isolated from *Stylosanthes* spp. while Peres et al. (2002) made similar observations with size ranging from 9.6 - 20.6 x 3.4 - 8.2 of isolates from various fruits. Given such variations in conidial dimension this study identifies both isolates 3012a and 5030b as *C. gloeosporioides*.

Molecular characterisation based on PCR banding patterns for the ITS region of the rDNA confirmed that all the 40 isolates from PNG were *Colletotrichum* isolates since the ITS 1 and ITS 4 primers used were able to produce identical banding pattern across all isolates. Similar banding patterns for various segments of the ITS region were reported

for *Colletotrichum* isolates originating from coffee and other hosts (Mills et al., 1992; Buddie et al., 1999; Manaut et al., 2001; Abang et al., 2002; Peres et al., 2002). Furthermore the size of the PCR product (500 - 600 bp) reported in this study is also similar to those reported in these earlier studies.

Given that 3 species of *Colletotrichum* have been found on coffee the results of the PCR product analysis is not adequate to separate the 3 species, *C. kahawae*, *C. gloeosporioides* and *C. acutatum.* This is further complicated by the argument that *C. kahawae* should be considered as a sub-species of *C. gloeosporioides* group species (Sreenivasaprasad et al., 1993). Hence further characterisation by way of RFLP analysis revealed two distinct groups among the 40 isolates of which DNA sequence analysis resulted in the identification of the PNG isolates as *C. gloesporoides* and *C. acutatum*.

The 11 isolates that were identified as *C. acutatum* were difficult to identify initially on the basis of conidial morphology and size and colony characters. Sutton (1992) pointed out that one of the problems that could arise in trying to identify *C. acutatum* and *C. gloeosporioides* lies in the fact that there exist strains of the later species which are intermediate in conidial morphology and size and show variable colony characters. Because of this confusion the individual worker may identify his/her strains as either *C. acutatum* or *C. gloeosporioides* depending on the criteria that is considered most important. *C. acutatum* species identified in this study reflects the complexity of the problem discussed by Sutton. While the DNA sequence analysis indicate the isolates as being closely related to *C. acutatum* some of the cultural and morphological features easily fit the isolates into the broad category of *C. gloesporioides*. However, in this study the results of the DNA analysis have been accepted together with the consistency of slow growth rates and the absence of setae as characteristic features of *C. acutatum*. The study also accepts conidial dimensions from these isolates as features characteristic of *C. acutatum* on coffee in PNG although these do not fall within the range observed by Simmonds (1965) and Hindorf (1970).

C. acutatum has been isolated from high altitude coffee (Hindorf, 1970). This is also evident in this study where it was only found in parts of the upper highlands, i.e Southern Highlands, Enga and Western Highlands Provinces. On the other hand *C. gloeosporioides* was found in all the provinces and confirms itself as a species of common distribution.

While this study was able to reveal the presence of *C. gloeosporioides* and *C. acutatum* there is no evidence to indicate the presence of the CBD pathogen, *C. kahawae,* in PNG. For comparison purposes the common cultural and morphological features of *C. kahawae* are as follows; conidia straight, cylindrical, measuring 12.5-19x4um formed from the mycelium, colonies dense to floccose, pale chocolate brown, sclerotia absent, setae usually absent (Sutton, 1980). Waller et al. (1993) described the colony characteristics on 2% MEA as follows: *C. kahawae*, slow growing (2-4 mm d^{-1} at 25^{o}C), profuse olivaceous to greenish dark grey mycelium, no acervular conidiomata produced, sporulation occurs from simple hyphae. *C. gloeosporioides*, faster growing (3-6 mm d^{-1} at 25^{o}C), white to

pale grey mycelium, sporulation from acervuli or simple hyphae. At the molecular level Sreenivasaprasad, et al., (1993) found some variations in the DNA sequence of the ITS region for *C. gloeosporioides* isolates whereas for *C. kahawae* isolates there were no DNA sequence variations. None of the isolates from PNG resemble the descriptions of *C. kahawae*. Furthermore field observations during sampling failed to identify infection of young green berries which is characteristic of CBD. However, infection of ripening green berries was quite common. Since the CBD pathogen can also infect ripening green berries the widespread occurrence of it is often the cause of anxiety and confusion among coffee growers as to its true pathological cause. This study confirms that infections of ripening green berries in PNG are caused by *C. gloeosporioides* either alone or in combination with *C. acutatum* although the pathogenicity of the latter is a subject of further investigations.

Diagnosis of the cause of berry anthracnose in PNG in the future can be done easily and quickly by adopting the methodology followed in this study. The methodologies used for extracting DNA, amplifying the ITS region of the rDNA and determining RFLP banding patterns, and cloning for DNA sequencing is relatively simple and can be done in PNG using some of the existing basic research facilities such as the UNITECH Biotechnology Centre. The only task that cannot be performed in PNG is DNA sequence determination and will thus rely on services provided by overseas laboratories. Analysis of DNA sequence appears to be the most relevant technique for separating *C. kahawae* from the other species given that Sreenivasaprasad et al., (1993) reported 100% sequence homology among isolates of *C. kahawae*. Should the CBD pathogen enter the country it is possible to identify it within 2 - 4 weeks from the time of sample collection.

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