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Pathogenic and genetic variability among *Colletotrichum gloeosporioides* isolates from different yam hosts in the agroecological zones in Nigeria

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Abstract

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., is the most severe foliar disease of water yam (*Dioscorea alata*) worldwide. Population genetic analyses can yield useful insights into the evolutionary potential of *C. gloeosporioides* and thus lead to the development of appropriate disease management strategies. The genetic structure of *C. gloeosporioides* populations from yam and non-yam hosts in three agroecological zones of Nigeria was investigated. Microsatellite-primed polymerase chain reaction (MP-PCR), virulence phenotyping using five putative *D. alata* differentials, cross-inoculation tests, and the presence/absence of a *Glomerella* teleomorph in yam fields were used to infer the evolutionary potential of *C. gloeosporioides* on yam. We observed high genotypic diversity (GD = 0.99 to 1.00) for populations from all hosts and agroecological zones, with multiple pathogen genotypes in individual anthracnose lesions. Genetic differentiation was low among pathogen populations from different hosts ($G_{ST} = 0.10$, $\theta = 0.034$), and agroecological zones ($G_{ST} = 0.04$, $\theta = 0.018$), indicating limited host differentiation and significant gene flow. No evidence was found for the existence of *C. gloeosporioides* f. sp. *alatae* reported in previous studies. The fungus was recovered from several non-yam host species commonly found in yam fields but non-yam isolates caused only mild to moderate symptoms on yam. Eighteen *C. gloeosporioides* virulence phenotypes were identified among 217 isolates but there was a weak correlation ($r = 0.02$, $P = 0.40$) between virulence phenotype and MP-PCR haplotype. Consistent with the above findings, we observed for the first time the *Glomerella* teleomorph on anthracnose-infected yam plants in Nigeria, indicating that sexual recombination might play an important role in anthracnose epidemics on yam. The implications of these findings for *C. gloeosporioides* evolutionary potential and anthracnose resistance breeding are discussed.

Introduction

Food yam (*Dioscorea* spp.) constitutes a major staple throughout West and Central Africa. Nigeria alone produces about 75% of the world's total, which is estimated at over 39 million tons (FAO, 2003). Foliar anthracnose, caused by the fungus *Colletotrichum gloeosporioides* Penz. [teleomorph *Glomerella cingulata* (Stonem.) Spauld. and Schrenk], is a damaging disease on yam, with water yam (*Dioscorea alata*) being particularly susceptible (Abang et al., 2002). The disease affects leaves, petioles and stems of yam plants, resulting in extensive necrosis of the foliage (Winch et al., 1984; Green, 1998). Severe losses in water yam production have been reported from West Africa, the Caribbean, India and the South Pacific (Winch et al., 1984; McDonald et al., 1998). Resistant yam cultivars could potentially form the basis of sustainable management strategies for anthracnose (Mignouna et al., 2001,2002); however, knowledge of *C. gloeosporioides* population genetics is needed to understand disease epidemiology, and to effectively breed and use resistant cultivars (McDonald and Linde, 2002).

Slow growing grey (SGG), fast growing salmon (FGS), and fast growing grey (FGG) strains of *C. gloeosporioides* have been described from yam in Nigeria

(Abang et al., 2001,2005). We used several *Colletotrichum* reference isolates and sequence analyses of the rDNA ITS region to show that only SGG and FGS isolates represent forms of *C. gloeosporioides* (Abang et al., 2002). It was considered that the FGG form probably represents a cryptic, but distinct, *Colletotrichum* species (Abang et al., 2002). Recent studies using denaturing gradient gel electrophoresis and microsatellite-primed PCR have shown that the SGG and FGS forms represent two genetically distinct pathogen populations on yam (Abang et al., 2005).

Considerable variation in virulence (=severity of disease) is known to exist among isolates of *C. gloeosporioides* from yam (Abang et al., 2001); however, little is known about pathotype diversity of the pathogen population in Nigeria, primarily because appropriate differential cultivars are lacking. Analysis of *C. gloeosporioides* from yam in Nigeria using RAPD markers revealed a high level of genetic variability and showed that some isolates from the same lesion are genetically different (Thottappilly et al., 1999). Studies of vegetative compatibility grouping (VCG) revealed high VCG diversity in *C. gloeosporioides* from yam, with multiple genotypes occurring in the same lesion (Abang et al., 2004a). The underlying mechanism producing such high levels of variability in this pathogen on yam in Nigeria is unclear. The sexual stage of *C. gloeosporioides* (*G. cingulata*) does not have a recognized role in anthracnose epidemics on yam, although it has been observed on heavily diseased senescent yam leaves in Guadeloupe (Toribio et al., 1980). Some *C. gloeosporioides* isolates from yam produce the *Glomerella* teleomorph on culture media (Abang et al., 2002) but there is no evidence that the fungus undergoes sexual reproduction on yam leaves in the field.

Weeds and crops such as *Citrus* spp. harbour populations of *C. gloeosporioides* that can be highly virulent on yam (Simons, 1993; Green, 1994). Simons (1993) found that isolates from lime (*Citrus aurantiifolia*) were highly virulent on *D. alata* (cv. White Lisbon) and argued that there was no justification for using the epithets f. sp. *alatae* and f.sp. *Dioscoreae* within *C. gloeosporioides* as suggested by Singh and Prasad (1967). The fact that *C. gloeosporioides* has been recovered from a large number of cultivated and wild plant species, many of which can be found in close proximity with yam, has led to suggestions that these hosts may serve as inoculum sources, and may pose a threat to commercial yam plantings in Nigeria (Abang et al., 2001). It has been shown that anthracnose disease is most prevalent and severe in the humid forest, followed by the derived savanna and Guinea savanna agroecological zones (Green, 1998), and one hypothesis is that *C. gloeosporioides* populations in these zones are genetically differentiated. Combining neutral molecular markers with pathogenicity assays represents a potentially useful method of investigating spatial and host differentiation in *C. gloeosporioides* (Freeman et al., 1996).

Genetic information required to design and deploy disease management tools can be obtained using a wide array of molecular techniques, including microsatellite-primed PCR (Abang et al., 2005). The objectives of the present study were to (i) assess the virulence spectrum on *D. alata* and the cross-infection potential of Nigerian isolates of *C. gloeosporioides*, (ii) investigate the presence of the *Glomerella* teleomorph in yam fields in Nigeria and (iii) use MP-PCR to assess the amount and distribution of genetic variation within and among populations of *C. gloeosporioides* originating from different yam species and non-yam hosts in three agroecological zones of Nigeria.

Materials and Methods

Origin of *Colletotrichum gloeosporioides* isolates

We surveyed 217 single-spore isolates of *C. gloeosporioides* collected from the humid forest zone, the derived savanna zone, and the Guinea savanna zone of Nigeria for their genetic diversity and virulence spectrum on yam (Fig. 1). The humid forest zone extends about 50–250 km along the coast with over 1500 mm of rainfall annually and a growing period of over 210 days. The derived savanna is characterized by 1200–1500 mm annual rainfall and a growing period of 181–210 days, while the Guinea savanna, extending North into the sahel savanna, has 900–1500 mm rainfall and a growing period of 150–210 days. To determine the diversity within and between individual fields, *C. gloeosporioides* isolates were collected from yam

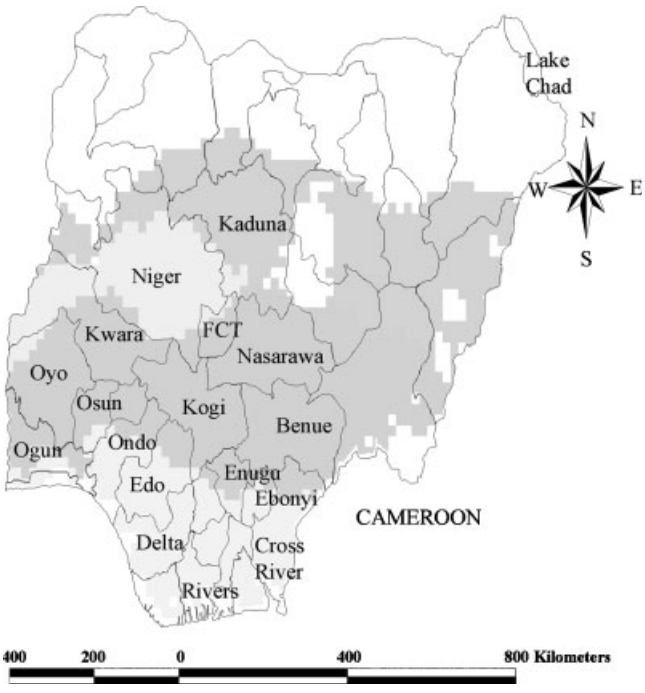

Fig. 1 Map of Nigeria showing the three-agroecological zones where *Colletotrichum gloeosporioides* isolates were collected. Collection sites in the federal states of Kaduna and Niger are located in the Guinea savanna, sites in the Federal Capital Territory (FCT), Oyo, Kwara, Osun, Kogi, Nasarawa, Benue, Enugu and Ebonyi states are in the derived savanna, while sites in Ogun, Ondo, Edo, Delta, Rivers and Cross River states are in the humid forest agroecological zone

fields in Ibadan and Mokwa. In addition, 15 *C. gloeosporioides* isolates from seven individual lesions (six pairs and one set of three isolates) from different yam plants were included in the study. *Colletotrichum gloeosporioides* isolates from *D. alata*, *Dioscorea rotundata*, *Dioscorea dumetorum*, *Dioscorea esculenta*, and some non-yam hosts were evaluated to determine the existence of host-associated pathogen populations, and to ascertain if such populations are genetically differentiated. A global positioning system receiver (Magellan GPS NAV DLX-10^{TM}, Magellan Systems Corporation, 960 Overland Court, San Dimas, CA 91773, USA) was used to record the co-ordinates of each sample collection point.

Virulence phenotyping

Five *D. alata* cultivars that differ in their response to individual *C. gloeosporioides* isolates and thus carry different genes for resistance to *C. gloeosporioides* (Abang et al., 2001; Mignouna et al., 2001,2002) were used for virulence phenotyping of 217 isolates. Minisetts from *D. alata* cultivars TDa 289, TDa 87/01091, TDa 85/00250, TDa 95/00328 and TDa 92-2 were planted into 18 cm diameter Steward plant pots (Steward product, England), containing 2.5 kg (dry weight) of sterilized soil. Plants were grown in a screenhouse under natural daylight, with temperatures ranging from 19 to 35°C. Young, newly but fully expanded, leaves were detached and inoculated following the method of Green et al. (2000). Briefly, the isolates were grown on potato dextrose agar (PDA) treated with streptomycin (300 ppm) at 28°C under an alternative 12-hour fluorescent light (60 $\mu E/m^2/s$)/12-hour dark to limit mycelial growth and induce sporulation. Seven-to-ten-day-old plate cultures were flooded with sterile distilled water supplemented with the wetting agent Tween 80 (2% v/v) and conidia were gently scraped off the plates. Spore density was adjusted with a haemocytometer and inoculum concentration was 10^6 spores per millilitre. Symptomless leaves were surface-sterilized by immersion in a 20% solution of commercial sodium hypochlorite for 2 min, followed by two rinses in sterile distilled water, each of 1 min. The leaves were placed on sterile paper towels in a laminar flow cabinet in order to remove excess moisture prior to inoculation. A fine-haired artists' paintbrush was used to apply newly prepared inoculum onto both leaf surfaces. The layout was a completely randomized design with three leaves per yam genotype.

Leaf area affected by anthracnose was scored using the individual leaf method for scoring anthracnose severity, in which inoculated leaves were rated for disease severity on a 0–6 scale (1 = 1%, 2 = 2%, 3 = 5%, 4 = 10%, 5 = 25%, 6 > 50%) identified using a visual key (Simons and Green, 1994). The average of three disease severity scores for each isolate was taken as the mean leaf area damage on a particular yam cultivar. Yam genotypes with a mean leaf area damage of 0–17.5% (corresponding to scores 0–4) were considered to be resistant, while those with a mean leaf area damage of >17.5% (corresponding to scores 5 and 6) were considered susceptible (Mignouna et al., 2001). Similarly, isolates resulting in <17.5% leaf area damage were considered as avirulent to a particular cultivar, while those that gave >17.5% leaf area damage were considered virulent. Reference virulent (Cg14 and Cg25) and avirulent (Cg15 and Cg40) isolates characterized in previous studies (Green et al., 2000) were used as positive and negative controls, while mock inoculations with Tween 80-amended sterile distilled water served as an additional check. Due to space constraints, independent experiments (runs) were performed on separate days but the positive and negative controls and mock inoculations with sterile distilled water were identical across runs. The differential reaction of each of the 217 isolates on the five *D. alata* cultivars was used to assign the isolates to the different virulence phenotypes or pathotypes.

Cross inoculation

Detached young leaves of yam (*D. alata* cv. TDa 92-2), mango, citrus, avocado and papaya were used to assess the cross-infection potential of selected isolates of *C. gloeosporioides*. The isolates were Cg14, Cg25, Cg33, Cg49 (yam), Cg 134, Cg140 (mango), Cg211, Cg214 (avocado), Cg54 (papaya), Cg194 (citrus) and Cg240 (cassava). Leaves were surface-sterilized, inoculated and incubated as described above. All possible treatment combinations were tested, each with five replications. Leaves were incubated for 7 days and assessed for disease severity using a scale of 0 for absence of symptoms; mild, <5% necrotic tissue; moderate, 5–50% necrotic tissue; severe, 51–100% necrotic tissue. When symptoms became visible, fungal isolations were made onto PDA plates and *C. gloeosporioides* isolates obtained were compared with the isolate used for inoculation based on colony and conidial morphology.

Occurrence of the *Glomerella* teleomorph

To investigate the occurrence of the sexual stage on yam in the field, a survey of different locations in Nigeria was conducted late (November) in the 2001 growing season. Samples of yam leaves with typical anthracnose symptoms (Winch et al., 1984), and of severely attacked and senescent yam leaves were collected and brought to the laboratory. Leaves were incubated under ambient laboratory conditions in large petri dishes lined with moist sterile paper towels. Leaves were examined regularly and fruit bodies were picked off with a needle under a stereomicroscope from a number of randomly selected microscope fields, along the length of the leaf. The fruit bodies were collected on a microscope slide in a drop of cotton blue (0.5% in a 1 : 1 : 1 mixture of lactic acid, glycerol and water), crushed and identified based on the spore morphology. For randomly chosen perithecia, measurements of asci (400 × magnification) and ascospores (1000×) were performed under the light microscope.

MP-PCR analysis

DNA extraction A subset of 122 representative isolates was selected based on the results of the virulence phenotyping and used in MP-PCR analysis. Total genomic DNA was extracted from aerial mycelium/conidia scraped off 7-day-old cultures using a modified cetyltrimethylammonium bromide (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., San Francisco, CA, USA).

PCR amplification MP-PCR analysis was carried out as described by Abang et al. (2005). Controls lacking fungal DNA were conducted for each experiment to check for DNA contamination of the reagents. Amplification products were separated by electrophoresis in 2% agarose gels.

Data analysis Analysis of the MP-PCR data was essentially as reported in Abang et al. (2005). MP-PCR products were scored as genetic data with positive (band present) and negative (band absent) alleles at 52 putative genetic loci. Both polymorphic and monomorphic markers were included, as recommended by Nei (1987). The number of MP-PCR genotypes, the frequency of the most frequent genotype and genotypic diversity (GD) (Hoffmann, 1986) were estimated using Multilocus 1.2 (Agapow and Burt, 2001). Estimates of the three parameters were compared with expectations under the null hypothesis of complete panmixia. The three diversity indices were calculated for each of 1000 randomizations to generate null distributions, and P values were estimated as 1-tailed statistics to test whether observed values were smaller or larger than expected in a randomized population. The analyses of H_S, G_{ST}, and Nei's genetic identity and genetic distance between and among pathogen populations were performed using the computer software POPGENE (Yeh and Boyle, 1997). Multilocus 1.2 was also used to estimate genetic differentiation using Weir's formulation of Wright's F_{ST} for haploids (Weir, 1996).

Gene flow between populations (Nm), where N is the population size and m is the fraction of individuals in a population that are migrants, was estimated by substituting Nei's G_{ST} for F_{ST} in Wright's model of gene flow (Wright, 1951) using a corrected version for haploids thus: $Nm = 0.5[(1–G_{ST})/G_{ST}]$ (McDermott and McDonald, 1993). If $Nm < 1$, then local populations tend to differentiate; if $Nm \geq 1$, then there will be little differentiation among populations and migration is more important than genetic drift (Wright, 1951).

A pairwise similarity matrix was compiled by the NTSYS-PC 2.0 software packages (Rohlf, 1997), using the Jaccard coefficient of similarity. A dendrogram was then constructed by UPGMA cluster analysis (Sneath and Sokal, 1973), in order to illustrate genetic relationships among *C. gloeosporioides* from different yam hosts. To measure the association between virulence phenotype and genotype, a simple Mantel test was conducted by comparing the matrix of genetic distances based on disease scores (pathotype) to that based on MP-PCR haplotype using the software zt (Bonnet and Van de Peer, 2002). The two distance matrices should be similar if virulence phenotype accurately depicts the relationships among isolates as estimated by the molecular data. The null hypothesis of no association between elements in the two matrices was tested by randomly rearranging the rows and columns of the distance matrices 1000 times and calculating a correlation coefficient (r value) after each iteration. The null hypothesis was rejected if fewer than 50 of the simulated r values exceeded the observed r ($P = 0.05$).

Results

Sexual reproduction

Investigations on the occurrence of the sexual stage *G. cingulata* revealed the abundance and predominance of *C. gloeosporioides* acervuli in anthracnose lesions of naturally infected *D. alata* leaves. The *G. cingulata* teleomorph was observed in lesions on severely infected senescent leaves obtained from Ibadan (Oyo state) and Idah (Kogi state). Perithecia, asci and ascospores had the distinctive morphology of *G. cingulata* and were typically found in association with conidia of *C. gloeosporioides*. Ascomata were rare and mostly remained immature, with few developing to maturity. All sexual structures fitted the description of Mordue (1971), and were similar in morphology to those observed previously in culture (Fig. 2) (Abang et al., 2002).

Virulence phenotyping

The leaf area affected by anthracnose was used to define compatible fungus/host interactions, and was scored with a rating scale for disease evaluation (Simons and Green, 1994; Green et al., 2000). The positive and negative checks, as well as the uninoculated control, gave a consistent disease reaction across replications and experimental runs, thus enabling comparisons to be made across runs. A whole plant assay showed a significant correlation with the detached leaf technique based on the cut-off value for susceptibility or virulence used in this study (Green et al., 2000), thus confirming that the detached leaf assay was an accurate indicator of cultivar susceptibility. All reaction types were found on the five yam cultivars used as differential hosts. Some fungal isolates induced no symptoms at all while others caused severe symptoms often with abundant sporulation. Yam cultivar TDa 92-2 was most susceptible to *C. gloeosporioides* infections with 80% of all fungal isolates being virulent on this cultivar (Table 1). Only about 40% of the isolates were virulent on TDa 95/00328 and TDa 87/01091, whereas 16 and 20% of the isolates were virulent on TDa 289 and TDa 85/00250, respectively. The 217 *C. gloeosporioides* isolates were grouped into 18 pathotypes based on the reaction patterns of the yam differential cultivars (Table 1). Pathotype 1, representing

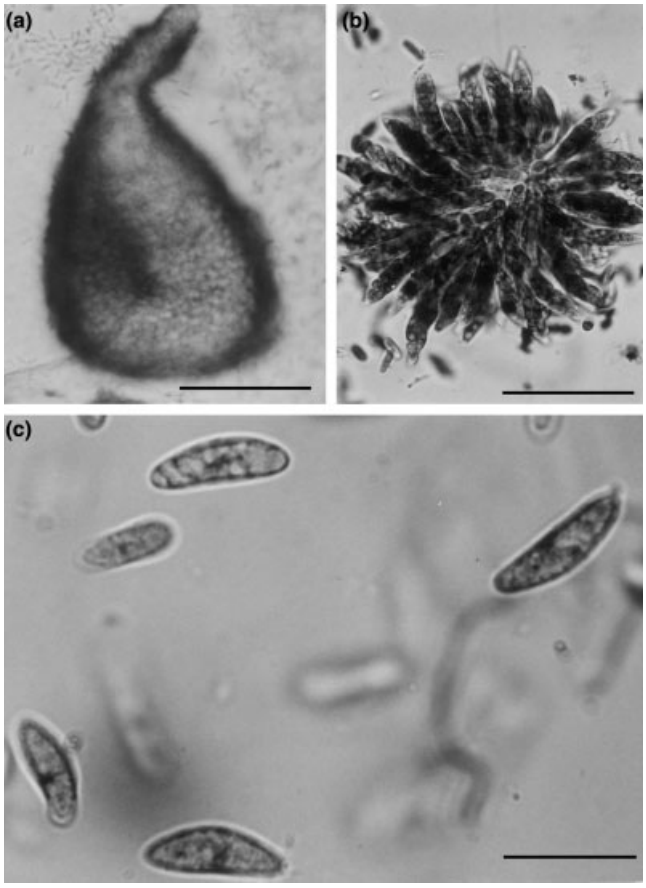

Fig. 2 Sexual structures of *Glomerella cingulata*: (a) mature perithecium, scale bar = 125 μm; (b) asci containing ascospores, scale bar = 45 μm; (c) mature ascospores, scale bar = 12 μm

2% of the isolates, comprised isolates that were virulent on all hosts. Interestingly, all these isolates were obtained from infected yam plants in IITA research fields. The most common fungal isolates, pathotypes 15 and 18, representing 22 and 11% of the isolates, respectively, were predominantly avirulent isolates.

Cross-infection potential

When *C. gloeosporioides* isolates from yam were used to inoculate other hosts, moderate symptoms were observed upon inoculation of papaya, avocado and mango (Table 2). Yam isolates did not infect citrus, and the citrus isolate 194 did not infect yam. Mango isolates caused mostly moderate disease reactions on yam, avocado and mango. Moderate disease reactions with typical anthracnose symptoms were induced in the susceptible yam cultivar TDa 92-2 upon inoculations with fungal isolates from other hosts, indicating the ability of *C. gloeosporioides* from other hosts to infect yam. Isolates obtained from *D. rotundata* (e.g. Cg33) caused severe disease symptoms on *D. alata*.

Table 2
Disease reactions of one yam and four non-yam hosts to eleven *Colletotrichum gloeosporioides* isolates from different hosts

Isolate desig-nation and original host	Disease severity[a]				
	Dioscorea alata cv. TDa 92-2	Citrus	Papaya	Avocado	Mango
Cg14, yam	+ + +	0	+	+	+ +
Cg25, yam	+ + +	0	+	+ +	+ +
Cg33, yam	+ + +	0	+	+ +	+ +
Cg49, yam	+ +	0	+ +	+	+ +
Cg134, mango	+	0	0	+ +	+ +
Cg140, mango	+ +	0	0	+	+ +
Cg211, avocado	+ +	+	0	+ +	+ +
Cg214, avocado	0	0	0	+ +	+ +
Cg54, papaya	0	0	+ +	+	+
Cg194, citrus	0	+	0	0	+
24, cassava	+	0	0	0	0

[a]Disease severity was assessed using the following scale: 0, no macroscopic symptoms; +, < 5% tissue necrotic (slight); + +, 5–50% tissue necrotic (moderate); + + +, 51–100% leaf tissue necrotic (severe).

Genetic structure based on MP-PCR analysis

Fifty-two scorable bands were generated by the four microsatellite primers and revealed a high level of polymorphism (Fig. 3). The four MP-PCR markers identified 36 and 46 polymorphic loci in the Ibadan and Mokwa populations, respectively (Table 3). Both fields had the maximum number of different genotypes (Table 3) with a frequency of the most frequent genotype of 1 for both sites, indicating that every strain represented a distinct haplotype. Average gene diversity across all loci was high for both fields (Table 3) but low G_{ST} (< 5%) and θ (0.018, $P = 0.14$) values indicated a high genetic similarity of *C. gloeosporioides* populations from yam at both sites. A further indication of the genetic similarity among geographic populations is provided by Nei's measure of genetic

Table 1
Virulence spectrum of 217 *Colletotrichum gloeosporioides* isolates using five putative *D. alata* differential cultivars

Cultivar	Number (%) of virulent isolates	Virulence phenotype/pathotype[a]																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TDa 92-2	173 (80%)	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	R	R
TDa 95/00328	83 (40%)	S	S	S	S	R	S	R	S	R	S	S	R	R	R	R	S	R	R
TDa 87/01091	89 (42%)	S	S	S	R	S	S	S	R	S	R	S	S	R	R	R	R	S	R
TDa 85/00250	43 (20%)	S	S	R	S	S	R	S	R	R	R	R	R	S	R	R	R	R	R
TDa 289	34 (24%)	S	R	S	S	S	R	R	S	S	R	R	R	R	S	R	R	R	R

[a]The differential reaction of each of the 217 isolates on the five *Dioscorea alata* cultivars was used to assign the isolates to the different virulence phenotypes or pathotypes. S, susceptible; R, resistant (Mignouna et al., 2001).

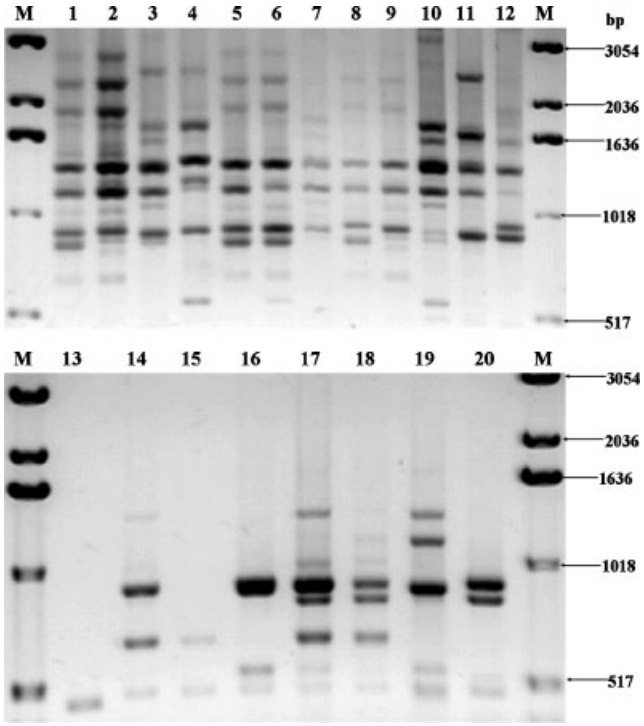

Fig. 3 Ethidium bromide-stained agarose gel, illustrating the DNA banding pattern of *Colletotrichum gloeosporioides* isolates using microsatellite-primed PCR primer $GACA_4$. The isolates represent collections from *Dioscorea alata* (lanes 1, 4, 6, 8–12 and 15–19), papaya (lane 5), *Dioscorea dumetorum* (lane 13), and *Dioscorea rotundata* (lanes 2, 3, 7, 14 and 20). $M = 1$ kb molecular size marker (Gibco BRL)

identity, $I = 0.98$. This indicates a 98% chance of choosing the same allele at any locus in a random draw of two individuals from these populations. The low average gene diversity between the two sites ($D_{ST} = 4\%$ of the total gene diversity) indicated that most of the genetic diversity is found within each subpopulation, with little diversity between subpopulations.

Table 4
Pairwise comparisons among *Colletotrichum gloeosporioides* populations from different agroecological zones in Nigeria

Population	Guinea savanna	Forest
Derived savanna	0.989[a]	0.990
	0.010[b]	0.009
	0.017 (P = 0.04)[c]	0.009 (P = 0.8)
Guinea savanna	–	0.978
	–	0.022
	–	0.031 (P = 0.05)

[a]Nei's (1978) genetic identity (*I*).
[b]Nei's (1978) genetic distance (*D*).
[c]Weir's (1996) coefficient of differentiation (θ) and P-value.

GD and numbers of different genotypes were high when *C. gloeosporioides* populations from the three agroecological zones were compared (Table 3). The frequency of the most frequent genotype was very low (ranging from 1 to 2). Average gene diversity was moderately high ($H_S = 0.26$); most of the genetic diversity was within the subpopulations (96%) with only 4% accounting for differences between populations from the different zones. G_{ST} and genetic distance values were low (< 5%), but pairwise comparisons of θ values revealed a marginally significant differentiation between the Guinea savanna population and those of the derived savanna and forest (Table 4).

Evaluation of the genetic diversity of *C. gloeosporioides* isolates from yam and other hosts revealed high GD (Table 3) and a low frequency of the most

Table 3
Genetic diversity within and between *Colletotrichum gloeosporioides* populations based on 52 microsatellite-primed PCR markers

Population	*n*	Ngen (P)[a]	Fgen (P)[b]	GD (P)[c]	NPL[d]	% PL[e]	H_T^f	H_S^g	D_{ST}^h	G_{ST}^i	Nm^j
Guinea savanna	43	43 (1.00)	1 (1.00)	1.00 (1.00)	48	92.3					
Derived savanna	33	32 (< 0.001)	2 (< 0.001)	0.99 (< 0.001)	50	96.1					
Forest	17	17 (1.00)	1 (1.00)	1.00 (1.00)	48	92.3					
Agroecologies	93	90 (< 0.001)	2 (< 0.001)	0.99 (< 0.001)	52	100.0	0.27	0.26	0.01	0.04	12.67
Dioscorea alata	39	39 (1.00)	1 (1.00)	1.00 (1.00)	51	98.1					
Dioscorea rotundata	44	42 (< 0.001)	2 (< 0.001)	0.99 (< 0.001)	46	88.5					
Dioscorea dumetorum	9	9 (1.00)	1 (1.00)	1.00 (1.00)	39	75.0					
Dioscorea spp.	92	89 (< 0.001)	2 (< 0.001)	0.99 (< 0.001)	51	98.1	0.27	0.25	0.02	0.05	8.85
Mango	9	9 (1.00)	1 (1.00)	1.00 (1.00)	31	59.6					
Citrus	8	8 (1.00)	1 (1.00)	1.00 (1.00)	36	69.2					
All hosts	109	104 (< 0.001)	2 (< 0.001)	0.99 (< 0.001)	51	98.1	0.26	0.23	0.03	0.10	4.56
Ibadan	17	17 (1.00)	1 (1.00)	1.00 (1.00)	36	69.2					
Mokwa	18	18 (1.00)	1 (1.00)	1.00 (1.00)	46	88.5					
Ibadan vs. Mokwa					51	98.1	0.24	0.23	0.01	0.04	12.46

[a]Total number of different genotypes.
[b]Frequency of the most frequent genotype.
[c]Genotypic diversity.
[d]Total number of polymorphic loci.
[e]Percent polymorphic loci.
[f]Gene diversity totalled among subpopulations.
[g]Gene diversity between subpopulations.
[h]Gene diversity within subpopulations.
[i]Genetic differentiation between subpopulations.
[j]Number of migrants.

frequent genotype (ranging from 1 to 2). Average gene diversity was moderately high for the host populations ($H_S = 0.23$), and was slightly higher when only *Dioscorea* hosts were considered ($H_S = 0.25$) (Table 3). Population differentiation was low among pathogen populations from different yam species ($G_{ST} = 0.05$); however, Nei's genetic distance and θ values revealed a moderate differentiation between *C. gloeosporioides* populations from citrus and those from *D. alata*, *D. dumetorum* and mango (Table 5). A graphical presentation of genetic similarity among different *Dioscorea* populations was obtained by generating a dendrogram from the similarity matrices based on Jaccard's coefficient and the UPGMA clustering method (Fig. 4). *Colletotrichum gloeosporioides* isolates obtained from the same yam species did not cluster together on the dendrogram, indicating that pathogen populations on different yam hosts are not genetically differentiated. Single spore isolates (e.g. Cg177a and Cg177b) obtained from the same lesion were found to be genetically different (Fig. 4).

Matrix correlation analysis using a simple Mantel test showed that there was a very weak and non-significant correlation between pathotype and MP-PCR haplotype ($r = 0.02$, $P = 0.402$).

Discussion

Our primary aim was to investigate the genetic structure of Nigeria isolates of *C. gloeosporioides* collected from different agroecological zones and from a variety of yam and non-yam host species. We were interested in demonstrating the presence of the *Glomerella* teleomorph on yam, and in determining whether levels of genetic diversity correlated with the apparent presence of a sexual stage in Nigeria. We also wanted to determine whether *C. gloeosporioides* isolates obtained from other hosts were capable of infecting water yam (*D. alata*) and whether these isolates were similar to those recovered from *D. alata*, because *D. alata* is reportedly attacked by a specialized form, *C. gloeosporioides* f. sp. *alatae* (Singh and Prasad, 1967).

Leaf area damage thresholds depicted on a visual scale were used to classify symptom ratings into compatible or incompatible interactions (Kelemu et al., 1999). Although the variation for anthracnose resistance is continuous, a gene-for-gene relationship was assumed (Mignouna et al., 2001,2002). All cultivars showed resistance to some isolates and no cultivar could be considered a universal suscept. No two cultivars reacted in the same manner to all isolates, apparently confirming that the test cultivars carried different resistance genes. Eighteen pathotypes were identified among 217 *C. gloeosporioides* isolates. This pathotype diversity is in agreement with Kelemu et al. (1996), who identified 57 pathotypes among 104 *C. gloeosporioides* isolates using 12 *Stylosanthes guianensis* differentials. It is likely that a larger differential set would have revealed more pathotypes. The high pathogenic variation among isolates of *C. gloeosporioides* should be considered when choosing isolates to use in an anthracnose screening program. It is obvious that no single isolate will suffice for resistance screening. Pathotypes 1–5 that caused susceptible reactions on most differential cultivars will be rational choices for an initial screening process. The inclusion of pathotype 4 isolates in disease screening is particular useful because previous studies on whole plants showed that this pathotype causes leaf abscission and premature death of susceptible yam cultivars (Mignouna et al., 2001).

The weak correlation between *C. gloeosporioides* pathotype and MP-PCR haplotype indicates a lack of association between genetic polymorphism and virulence. The high GD observed in this study and by Thottappilly et al. (1999), and the occurrence of the sexual stage of *C. gloeosporioides* in yam fields in Nigeria are consistent with the observation that isolates of the same pathotype may not be necessarily closely related. If pathotypic changes are slow, as is probably the case with the yam anthracnose pathogen, then random molecular variations will accumulate in every pathotype resulting in little correlation between pathotype and lineage. The absence of a strong link between genetic and pathogenic groupings observed in this study agrees with findings on the *Stylosanthes* - *C. gloeosporioides* pathosystem (Kelemu et al., 1997,1999; Weeds et al., 2003).

Table 5
Pair-wise comparisons among yam and non-yam host populations of *C. gloeosporioides* in Nigeria

Population	*Dioscorea rotundata*	*Dioscorea dumetorum*	Citrus	Mango
Dioscorea alata	0.984[a]	0.980	0.965	0.986
	0.016[b]	0.020	0.035	0.014
	0.026 (P = 0.62)[c]	0.032 (P = 0.1)	0.064 (P = 0.01)	0.009 (P = 0.59)
D. rotundata	–	0.978	0.972	0.976
	–	0.022	0.028	0.024
	–	0.025 (P = 1.0)	0.004 (P = 0.26)	0.033 (P = 1.0)
D. dumetorum		–	0.942	0.970
		–	0.059	0.030
		–	0.086 (P = 0.04)	0.037 (P = 1.0)
Citrus			–	0.952
			–	0.049
			–	0.091 (P = 0.03)

[a]Nei's (1978) genetic identity.
[b]Nei's (1978) genetic distance.
[c]Weir's (1996) coefficient of differentiation (θ) with P-value.

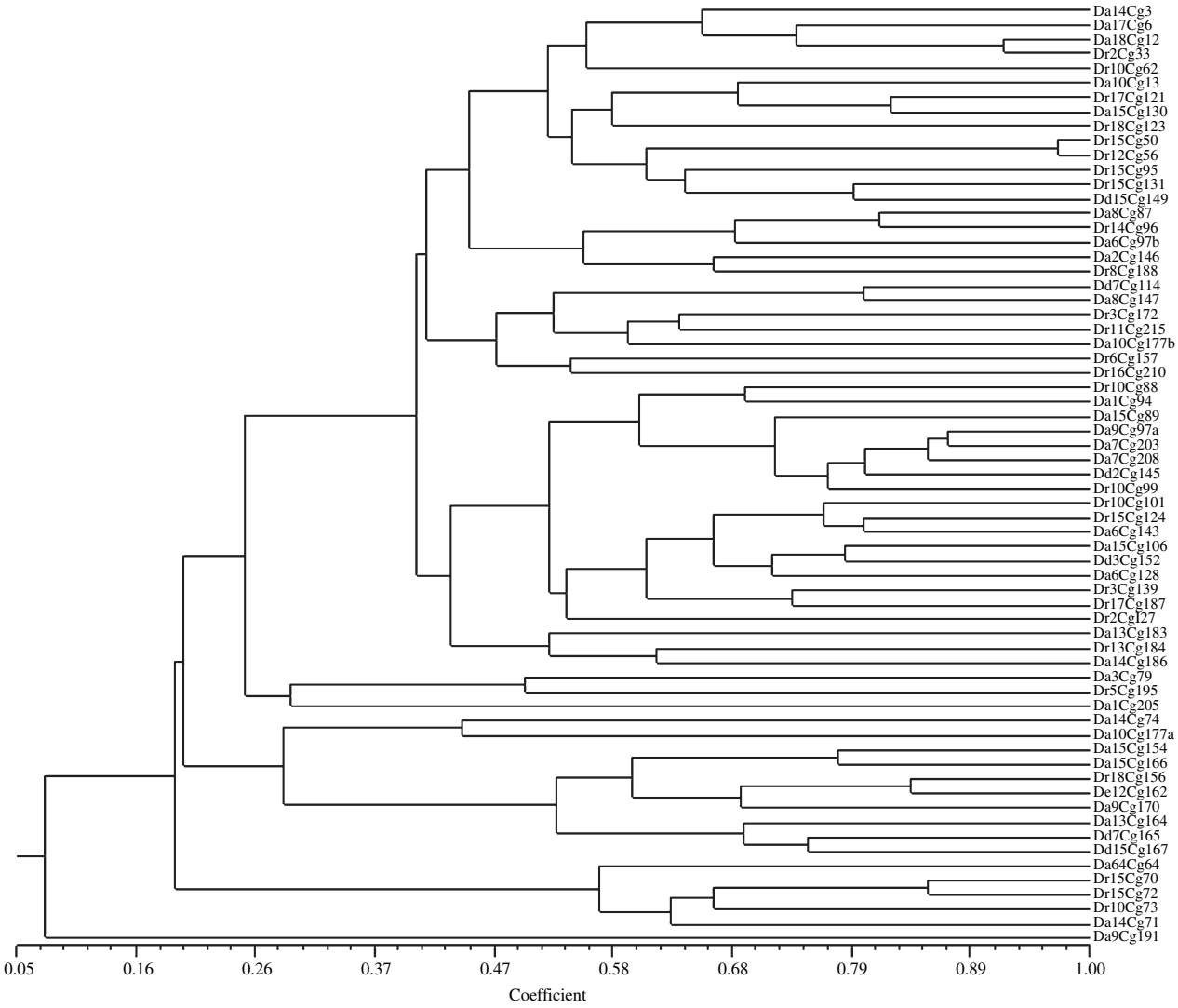

Fig. 4 Dendrogram illustrating genetic relationships among *Colletorichum gloeosporioides* isolates sampled from different *Dioscorea* species in Nigeria based on microsatellite-primed PCR analysis. The isolates are designated by alphanumerics indicating the *Dioscorea* species (Da, Dr, Dd and De represent *Dioscorea alata*, *Dioscorea rotundata*, *Dioscorea dumetorum*, and *Dioscorea esculenta*, respectively), followed by the pathotype number, e.g. 14 for pathotype 14, and then isolate number, e.g. Cg3. Isolates Cg177a and Cg177b are cultural variants isolated from the same lesion

A low level of genetic differentiation by host was found among *C. gloeosporioides* isolates sampled from three different *Dioscorea* species. This contradicts the idea that anthracnose disease on *D. alata* is caused by a specialized form of the pathogen, *C. gloeosporioides* f. sp. *alatae*, as proposed by Singh and Prasad (1967). When isolates were pooled by yam host, a G_{ST} of 0.05 was obtained, which is very small compared to 0.34 that has been reported for host-differentiated populations such as *Sclerotinia homoeocarpa* on turfgrass (Hsiang et al., 2000). A moderate population differentiation was observed between *C. gloeosporioides* isolates from citrus, and those from *D. alata*, *D. dumetorum* and mango, which reflected the results of the cross-inoculation tests. This suggests that isolates from citrus isolates do not pose a threat to *D. alata*; however, failure to obtain cross infection following inoculation of a single citrus genotype with a few *C. gloeosporioides* isolates cannot rule out the possibility that cross infection occurs in nature. Citrus in the Caribbean is known to harbour isolates of *C. gloeosporioides* that are highly virulent on yam (Simons, 1993; Green, 1994).

We observed, for the first time, the sexual stage of *C. gloeosporioides* (*G. cingulata*) on heavily diseased yam leaves collected from the field in Nigeria. GD was extremely high for all pathogen populations, which is not unusual for an organism that reproduces sexually. Seven of the eight lesions from which multiple isolations were made revealed the presence of more than one *C. gloeosporioides* genotype. In a related study, we found that isolates with different VCG genotypes were associated with individual lesions (Abang et al., 2004a). Thottappilly et al. (1999) also reported several examples of isolates with unique RAPD haplotypes originating from the same yam anthracnose lesion. Outcrossing that occurs in the unusual and complex mating system of *G. cingulata* (unbalanced heterothal-

lism) is thought to give rise to offspring with novel genetic combinations (Cisar et al., 1994). Mating in *G. cingulata* is controlled by a single locus with multiple alleles (Cisar et al., 1994). The occurrence of multiple genotypes in the same lesion allows isolates with complementary alleles at the mating type locus to come together and reproduce sexually. Ascomata were exclusively found on heavily diseased yam leaves with coalesced lesions, indicating the need for single lesions containing complementary mating types to coalesce prior to the initiation of perithecia. Frequent sexual reproduction in turn will ensure frequent recombination and increased evolutionary adaptability of the pathogen (McDonald and Linde, 2002).

If the primary infective propagules of *C. gloeosporioides* on yam in Nigeria were only conidia, pathogen populations would display widespread occurrence of identical genotypes and a high level of differentiation among geographically separated populations due to the limited long-distance spread of conidia dispersed by rainsplash (Wastie, 1972). Little evidence was found for clonal spread or population subdivision in this study, which is indicative of a genetically recombining population. Ekefan (1996) analyzed spatial patterns of yam anthracnose disease in Nigeria using an epidemiological approach and found that spread occurs from randomly distributed foci in fields. Random spatial patterns of disease, high GD, lack of association between MP-PCR haplotype and virulence phenotype, and the presence of sexual fruiting bodies on infected yam leaves are consistent with ascospore inoculum contributing to the epidemiology of anthracnose disease on yam (Milgroom and Peever, 2003).

The high genetic diversity reported in this study could also be explained by assuming that pathogenicity on yam may have been acquired by a large number of genetically distinct strains. West Africa is one of the centres of origin of yam (Coursey, 1973), and is thus a presumed centre of diversity of its pathogen *C. gloeosporioides*. This can influence pathogen variability because diversity of *C. gloeosporioides* has been shown to be extensive at sites where native or naturalized host populations occur compared to sites were the host has been introduced recently (Weeds et al., 2003). Akem and Asiedu (1994) found that yam was grown as an intercrop with an array of other crops in more than 77% of yam fields surveyed for anthracnose in Nigeria. The multitude of yam and non-yam hosts commonly found within the same field may have led to either the introduction of diverse *C. gloeosporioides* genotypes at each location and/or the maintenance of diverse genotypes.

Gene flow is another possible explanation for the high GD within populations and high genetic similarity among geographic populations in Nigeria (McDermott and McDonald, 1993). The average G_{ST} of 0.04 ($Nm = 12.67$) indicated little genetic diferentiation among *C. gloeosporioides* populations in the three agroecological zones. The G_{ST} for Ibadan and Mokwa (320 km apart) populations was also 0.04 ($Nm = 12.46$), providing further evidence for the high genetic similarity between field populations of the pathogen in Nigeria. While estimates of *Nm* obtained in this study may not exactly reflect the number of migrants that are exchanged between pairs of populations each generation (Keller et al., 1997), they indicate the relative degree of gene flow that likely occurred between populations over time (Keller et al., 1997). The *Nm* values of >12 suggest that there have been few restrictions to gene flow in the past among these populations. A high level of gene flow between geographically distant populations may unite them into homogeneous genetic groups that are evolving together. Isolates were grouped in this study based on agroecological zones or geographic separation, but the results of population genetic analyses suggest that these 'geographic populations' are not evolving independently and hence may be considered part of the same 'genetic population'.

Several mechanisms could facilitate gene flow between populations of *C. gloeosporioides*. The most obvious is air dispersal of the ascospores. The forcible discharge of ascospores is important in the dispersal of many plant pathogenic ascomycete fungi (Keller et al., 1997; Trail et al., 2002). Discharged ascospores become airborne and may serve as the primary inoculum for epidemics over considerable distances (Trail et al., 2002). Wastie (1972) observed that ascospores of *G. cingulata* on fallen twigs and leaves may under certain circumstances be of greater importance than *C. gloeosporioides* conidia in the spread of secondary leaf fall of rubber, especially under dry weather and at the start of the refoliation season. A similar situation probably exists in yam fields but this requires further investigation. Monitoring the pathogen population in the course of two or three growing seasons may reveal a period of ascospore discharge and the presence of ascospore showers.

Other mechanisms that may also facilitate gene flow are alternative hosts and infected tubers. Both *C. gloeosporioides* and its teleomorph, *G. cingulata* are generalists with broad host ranges. It is possible that one or more of the alternative hosts for *C. gloeosporioides* form a continuous host population throughout the yam production areas of Nigeria. If it is confirmed that conidia and ascospores move readily between local geographic populations of mango, rubber, papaya, sugarcane, avocado, weeds, etc., then these alternate hosts could maintain a uniform source population for the conidia/ascospore inoculum that infects yam fields each season. Abang et al. (2004a)) found that *C. gloeosporioides* isolates from yam were vegetatively compatible with weed isolates and hypothesized that genetic exchange may take place between isolates from yam and non-yam hosts under field conditions. The huge yam trade across Nigeria means that there are few restrictions to gene flow among geographic populations of the pathogen in the country.

The pyramiding of resistance genes has been suggested as a potentially valuable strategy in anthracnose resistance breeding (Mignouna et al., 2002).

However, this strategy is appropriate if the pathogen is exclusively asexual and if the potential for gene flow is low. But if the pathogen is recombining, as suggested by the findings of this study, then the recombination of virulence alleles may occur as quickly as breeders can recombine resistance genes, thus jeopardizing breeding efforts (McDonald and Linde, 2002). Also, the lack of genetic differentiation among host populations of *C. gloeosporioides* means that weeds and other hosts may represent significant sources of inoculum for anthracnose epidemics on yam (Milgroom and Peever, 2003). These conclusions should be treated with caution because of the relatively small number of isolates in some host populations studied. However, bootstrap tests of significance enabled us to estimate indices of diversity and population differentiation with a reasonable degree of confidence (Grünwald et al., 2003).

Indirect estimates of population genetic parameters and confirmation of the occurrence of a sexual stage indicate that *C. gloeosporioides* on yam in Nigeria is characterized by high genetic diversity, high gene flow and possible sexual recombination. These results provide justification for further investigation of *C. gloeosporioides* evolutionary potential using mark-release-recapture field experiments in order to provide a sound basis for advising breeders on the choice of an appropriate resistance breeding strategy (Abang et al., 2004b).

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