Uganda Journal of Agricultural Sciences, 2013, 14 (1): 111 - 123 ISSN 1026-0919

Genetic characterisation of Ugandan strains of *Colletotrichum sublineolum* using ISSR makers

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Abstract

***Colletotrichum sublineolum*, the causal agent of sorghum anthracnose, presents high variability, genetic instability and host specialisation, hence rapidly breaking down resistance. In Uganda, no population studies of *Colletotrichum sublineolum* have been reported hence there is limited information on the nature of epidemics and pathogen. The present study aims at investigating the genetic diversity within 124 *Colletotrichum sublineolum* isolates from Uganda. The inter-simple sequence repeats (ISSR) produced 37 polymorphic loci and neighbor-joining analysis revealed two major groups. There were no major groups among all isolates in studies. Analysis of molecular variation (AMOVA) based on 7 agro-ecologies of Uganda revealed the presence of a population structure, (ΦF_{ST} = 0.08 P = 0.05) leading to acceptance of null hypothesis stating a presence of population differentiation between the agro-ecologies in Uganda. Gene flow between agro-ecologies was 5.75 calculated from $\hat{O}F_{ST.}$ The results of the AMOVA analysis revealed the allelic variation (92.5%) was shared between populations. Average gene diversity over all loci ranged from 0.192 to 0.335 showing high diversity within population rather than between populations. The numbers of polymorphic loci were similar for the population studied.**

Key words: Colletotrichum sublineolum, ISSR, molecular markers, sorghum, strains

Introduction

Colletotrichum sublineolum is one of the most devastating diseases affecting cultivated sorghum (Sherriff *et al.,* 1995; Thakur and Mathur, 2000). The disease is known to occur in epidemic proportions in many parts of the world, causing severe losses depending on the crop stage and cultivar susceptibility. The severity and significance of damages caused by this pathogen necessitates development of strategies to control and manage the disease so as to reduce crop losses and avert epidemics. Host resistance has been used in the management of several diseases and thus breeding sorghum varieties with genetic resistance to sorghum anthracnose is an effective and economical strategy for control of the disease. *Colletotrichum sublineolum* is a highly variable pathogen that rapidly breaks down resistance (Thakur, 1995). Changes in the virulence pattern of

pathogen populations have been reported from several parts of the world.

The first report on existence of races of *C. sublineolum* was in 1967 (Thakur and Mathur, 2000). From 1967 to 1991, 44 *Colletotrichum* spp. races or pathotypes have been reported, but due to the use of different sets of host differentials and rating scales, a global picture of the pathogenic races is not clear (Thakur, 2004). The appearance of different pathogenic races could be explained by the long-time association of the pathogen with sorghum (Ali and Warren, 1987). Different symptoms produced on sorghum genotypes and differences in cultural characteristics suggest physiological variation within pathogen populations (Pande *et al.,* 1991). Increased variability of *C. sublineolum* has been attributed to the parasexual cycle (Souza-Paccola *et al.,* 2003). A gene-for-gene interaction between sorghum genotypes and pathogenic races has not been demonstrated (Rosewich *et al.,* 1998), although, it has been considered that the pathogen evolves alongside the traditional sorghum landraces, suggesting a gene-for-gene interaction (Ngugi *et al.,* 2002).

The rapid resistance break-down poses a great challenge to sorghum breeding. Therefore one way to reduce rapid resistance break down is to slow down the rate of evolution of novel genotypes through use of techniques that do not create very high selection pressure on the pathogen (McDonald and Linde, 2002). Since some resistant genes are effective only against particular pest or pathogen sub-populations, it is important to understand the structure of pathogen populations in order to determine the best strategy for deployment of resistance and/or incorporate the non-matching resistance genes to the existing pathogen. Consequently, information on pathogen population, diversity, and virulence will be very important for a successful sorghum-breeding program. Unfortunately, there is limited information regarding *Colletotrichum sublineolum* populations in Uganda, yet the disease continues to impede production both locally and elsewhere.

In this study, Colletotrichum isolates collected from 7 agroecologies were genotyped using ISSR markers. Recently, various types of DNA-based molecular techniques such as hybridisation, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for diversity studies (Joshi *et al*., 2004; Shcher and Carles, 2008). Among PCR-based methods, inter-simple sequence repeats (ISSRs) has been found to be an efficient and reliable technique established by Zietkiewicz *et al*. (1994) for the identification of species or varieties, population authentication and population genetic structure, etc. (Shen *et al*., 2006). Compared with random amplified polymorphic DNA (RAPD) method, amplified fragment length polymorphism (AFLP), and other molecular markers, the main advantages of ISSR are: no need for DNA sequence information prior to amplification, low cost, simple operation, high stability, and abundance of genomic information. In the present study, we examined the diversity of ISSR markers in Colletotrichum sublineolum and evaluated the genetic relationship among different isolates.

Materials and methods

Fungal culture

Diseased sorghum leaves were collected from sorghum fields in nineteen districts of Uganda where sorghum and/or maize are commonly cultivated (Table 1). Leaf samples were air dried for 7 days and stored at room temperature. Lesions which showed clear anthracnose symptoms were excised out from each leaf sample, surface sterilised using 0.5% sodium hypochlorite solution, rinsed twice in sterile distilled water, placed on moist filter paper in a Petri dish and incubated for 48 to 72 hours to stimulate sporulation. Conidia were dislodged from the lesions with the aid of stereo-microscope and then plated on Potato Dextrose Agar plates and cultured under natural light and darkness regimes for 48 to 72 hours at room temperature. Monoconidial cultures of the isolates were then established by sub-culturing germinated spore bearing distinct colony characteristics of *C. sublineolum* to fresh Potato Dextrose Agar plates (Difco Laboratories) amended with 100 mg ml^{-1} of ampicilin. To obtain mycelium for DNA extraction, 200mls of potato dextrose broth (Difco Laboratories, Becton Dickinson, Microbiology Systems, Sparks, MD, USA) was inoculated with culture plugs of *C. sublineolum* and cultured at room temperature (25 ^{o}C) for 8 days on a rotary shaker (Laboshake - laboratory shakers LS/RO 500 Gerhardt, Germany) Mycelium was harvested by vacuum filtration after 8 days to prevent generation of high molecular weight carbohydrates that compound DNA isolation (Okori *et al.,* 2003). Mycelial fragment were stored at -80 ^{o} C for DNA isolation.

Isolation and extraction of DNA

Genomic DNA extraction was performed by a modified extraction protocol (Okori *et al.,* 2003) using the hexadecyltrimethylammonium bromide (CTAB). Briefly, 500 mg of mycelial of each isolate were grain in a mortar using a pestle on to which liquid nitrogen was powered, and transferred to 50 ml microcentrifuge tubes containing 5 ml of extraction buffer (0.1 M Tris-HCl pH 8.0; 0.2 M NaCl; 0.02 M EDTA; 1,0% SDS; 0.1% b-mercaptoethanol). Each tube was vigorously agitated to obtain a uniform suspension without lumps and then incubated for 15 min at room temperature. Afterwards, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to each sample, vigorously agitated, and incubated for 10 min at room temperature. Cell debris was removed by centrifugation at 4 ^{O}C with 8000 rpm for 10 minutes. Aliquots of 5 ml of the supernatant layer were transferred to a 50 ml propilene tube and an equal volume of ice cold ethanol was added to each sample and gently inverted several times to precipitate nucleic acids. Nucleic acids were spooled out on a glass hook, briefly washed in 70% ethanol and dissolved with 0.5 ml of TE buffer. Genomic DNA was quantified for each sample by spectrometry and dilutions made to a final concentration of 20 ng.

Inter Simple Sequence Repeats (ISSR) analysis

Polymerase Chain Reaction amplification reactions were performed in a total volume of 15 ìL. Each reaction mixture contained about 20 ng of template DNA, 2.5 mM $MgCl_2$, 0.5 ìM each primer (Table 2), 0.2 mM each dNTP, and 1 U promega

Table 1. Origin and designations of the isolates used in the study

Isolate	District of origin	Isolate	District of origin
Amu5	Amuria	kabe19	Kaberamaido
Amu1	Amuria	kabe29	Kaberamaido
Amu16	Amuria	Kabe1	Kaberamaido
Amu4	Amuria	Kabe8	Kaberamaido
Amu15	Amuria	Lr9	Lira
Amu3	Amuria	Lr13	Lira
Amu2	Amuria	Lr1	Lira
Ap2	Apac	Lr15	Lira
Ap3	Apac	Lr12	Lira
Ap5	Apac	Lr3	Lira
Ap8	Apac	Lr11	Lira
Ap12	Apac	Lr8	Lira
Ap18	Apac	Lr10	Lira
Ap4	Apac	Lr18	Lira
Ar1	Arua	Lr3	Lira
Ar28	Arua	Ms18b	Masaka
Ar15	Arua	Ms19	Masaka
Ar12	Arua	Ms14	Masaka
Ar16	Arua	Ms17	Masaka
Bs1	Bushenyi	Ms15	Masaka
Bs3	Bushenyi	Ms11	Masaka
Bs4	Bushenyi	Ms2	Masaka
Bs9	Bushenyi	Ms22	Masaka
Bs19	Bushenyi	Ms8	Masaka
Bs6	Bushenyi	Ms17	Masaka
Bs14	Bushenyi	Ms5	Masaka
Bs2	Bushenyi	Ms18a	Masaka
Dok13	Dokolo	Ne9	Nebbi
Dok4	Dokolo	Ne8	Nebbi
Dok5	Dokolo	Ne25	Nebbi
Dok6	Dokolo	Ne1	Nebbi
Dok9	Dokolo	Ne3	Nebbi
Dok8	Dokolo	Ne13	Nebbi
Dok1	Dokolo	Ne11	Nebbi
Dok13	Dokolo	Ne17	Nebbi
Dok3	Dokolo	Ne14	Nebbi
Ho13a	Hoima	Ne22	Nebbi
Ho29	Hoima	Ne9b	Nebbi
Ho13b	Hoima	Ne16	Nebbi
Ho26	Hoima	Ne23	Nebbi
Ho27	Hoima	Ne15	Nebbi
Ho14	Hoima	Ne19	Nebbi
Ho12	Hoima	Ne18	Nebbi
Ho4	Hoima	Ne5	Nebbi
Ho6	Hoima	Ne7	Nebbi

Table 1. Contd.

Isolate	District of origin	Isolate	District of origin
Ho7	Hoima	St17	Soroti
Ho8	Hoima	St11	Soroti
Ho19	Hoima	St18	Soroti
Kabe18	Kaberamaido	Tr10	Tororo
Kabe21	Kaberamaido	Tr35	Tororo
Kabe2	Kaberamaido	Tr13	Tororo
Kabe7	Kaberamaido	Tr35	Tororo
Kabe25	Kaberamaido	Tr25	Tororo
Kabe17	Kaberamaido	Tr17	Tororo
Kabe3	Kaberamaido	Tr12	Tororo
Kabe21	Kaberamaido	Tr27	Tororo
Kabe2	Kaberamaido	Tr13	Tororo
Kabe14	Kaberamaido	Tr16	Tororo
Kabe20	Kaberamaido	Tr3	Tororo
Kabe4	Kaberamaido	Tr20	Tororo
Kabe22	Kaberamaido	Tr2	Tororo
Kabe7	Kaberamaido		

Table 2. Description of species specific primers used for PCR in this study

Primer	Sequence (5' – 3')	T_m (^{0}C)
DHB	CGACGACGACGACGA	50
BDB	ACAACAACAACAACA	40
DDC	CCACCACCACCACCA	50

T_m, = Melting temperature calculated from 4(G+C) + 2(A+T). All primers were supplied by Invitrogen Life Technologies

GoTaq DNA polymerase (Cape town, South Africa) in a reaction buffer containing 10 mM Tris-HCL pH 8.8, 50 mM KCl and 0.1% Triton X – 100.

Polymerase chain reaction amplification was performed in a thermocycler (Geneamp systems, 9700, Applied Biosystems 850 Lincoln Centre Drive | Foster City, CA 94404 USA) set to the following programmes. For primer BDB and DDC, 94^{0}C for 2 min, 35 cycles of 94^{0}C for 30 sec, 50^{0}C for 30 sec, 72^{0}C for 1 min, and a final extension of 72^{0}C for 10 min. For primer DHB, 94^{0}C for 2 min, 35 cycles of 94^{0}C for 1 min, 48^{0}C for 2 min, 72^{0}C for 2 min, and a final extension of 72^{0}C for 10 min. Control tubes containing master mix without template DNA were included in each run and the reactions were repeated at least three times to check the reproducibility of the process.

Amplification products were mixed with loading solution (dye) in the ratio of 1:5 and verified on 1% agarose gels stained with 10 mg mL^{-1} ethidium bromide in 1X Tris-borate EDTA (TBE) buffer. Electrophoresis was performed at 100 V

for 2 hours and the resulting DNA fragments visualised by UV light (BIORAD, Hercules, C.A, and U.S.A).

Data analyses

The amplification products were scored in terms of a binary code as present (1) or absent (0), each of which was treated as a unit character regardless of its intensity. Data generated from each primer combination were pooled to generate one multilocus data set comprising 37 fragments that was subsequently used in genetic analyses. Genetic parameters including the polymorphism (the proportion of polymorphic loci to the total number of loci scored in all samples of the three species) and Nei's genetic distance were calculated using the computer program Arlequin ver *3.5* (http://cmpg.unibe.ch/software/arlequin3). The obtained genetic distance matrix was then used to perform the cluster analysis and construct the UPGMA (unweighted pair group method with arithmetic mean) dendrogram using TREECON version 1.3b (Van de Peer and De Wachter, 1994). Robustness of the dendogram was tested by bootstrap analysis of 1000 replicates.

For population genetic analyses, the 123 fungal isolates were structured into eight populations divided by their agro-ecological zone of origin. Genetic variation in fungal collections was also measured by Nei's gene diversity, which measures the probability of obtaining two different alleles at a locus when two haplotypes are sampled from a population (Nei, 1973), and Nei's measure of genetic distance (Nei, 1987). Statistical comparisons of gene diversity estimates were performed using t-test (Nei, 1987). A null hypothesis indicating presence of population structure between populations was analysed using Wright's population differentiation index F_{st}. The fixation index as described by Wright equals to the reduction in heterozygosity expected with random mating at any one level of population hierarchy relative to another more inclusive level of hierarchy (Wright, 1987). In this study, ΦF_{st} was estimated using the Analysis of Molecular Variance (AMOVA) framework (Excoffier *et al.*, 1992) and implemented in Arlequin (Schneider *et al.,* 2000). AMOVA estimates fixation indices in form of $\Phi F_{st,}$ which attempts to correct for the effects of sampling a limited number of organisms from a limited number of populations. Theoretically, the fixation index has a maximum of 1 and a minimum of 0. Further tests for population differentiation were performed using exact tests at the 5% significance level as implemented in Arlequin (Raymond and Rousset, 1995). Significance of ΦF_{st} as estimated in AMOVA was tested using non-parametric permutation procedures in Arlequin (Schneider, Roessli and Excoffier, 2000). Gene diversity (i.e. the probability that two randomly chosen haplotypes are different in a sample) was also calculated. ($) and its sampling variance V ($) were computed as:

$$\hat{H} = \frac{n}{n-1}(1-\sum_{i=1}^{k} p_i^{\,2})$$

$$V(\hat{H}) = \frac{2}{n(n-1)}\left\{2(n-2)\left[\sum_{i=1}^{k} p_i^{\,3} -\right.\right.$$

$$\left.\left.(\sum_{i=1}^{k} p_i^{\,2})^2\right] + \sum_{i=1}^{k} p_i^{\,2} - (\sum_{i=1}^{k} p_i^{\,2})^2\right\}$$

Where *n* is the number of gene copies in the sample, *k* is the number of haplotypes

and *Pi* is the sample frequency of the i-th haplotype (Nei, 1987). The $ values were compared by t-test (Nei, 1987).

Results

Population study of *Colletotrichum sublineolum*

PCR of *Colletotrichum sublineolum.* A total number of bands within the range of 50 to 300 bp were scored for the ISSR analyses. Repetitions using the same set of primers and fungal isolates consistently yielded similar polymorphic band numbers and patterns (Fig. 1). This reproducibility was possible because primer annealing is very specific due to homology to both the adapter sequence and the restriction site sequence. Amplified fragments lower than 200 bp and faint bands were neglected, because the resolution was insufficient to discriminate between bands of various molecular sizes. These data were used for phylogenetic and population genetic analyses.

Population structure of *Colletotrichum sublineolum.* Analysis of molecular variation (AMOVA) of ISSR data on the basis agro-ecologies revealed the presence of a population structure, (ΦF_{ST} = 0.08 P = 0.05) leading to acceptance of null hypotheses stating a presence of population differentiation between the agro-ecologies in Uganda (Table 4) since than ΦF_{ST} was greater the 0. The results of the AMOVA analysis also revealed the allelic variation (92.5%) was shared between populations.

Table 3. Analysis of molecular variation *Colletotrichum sublineolum* from 7 Ugandan agroecological zones

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups	6	65.027	0.22795	4.06
Among population within groups	5	33.694	0.19321	3.44
Within population	104	539.624	5.18869	92.49
Total	115	638.345	5.60985	

ΦF_{ST} = 0.08 (P = 0.05) based on 1000 permutations

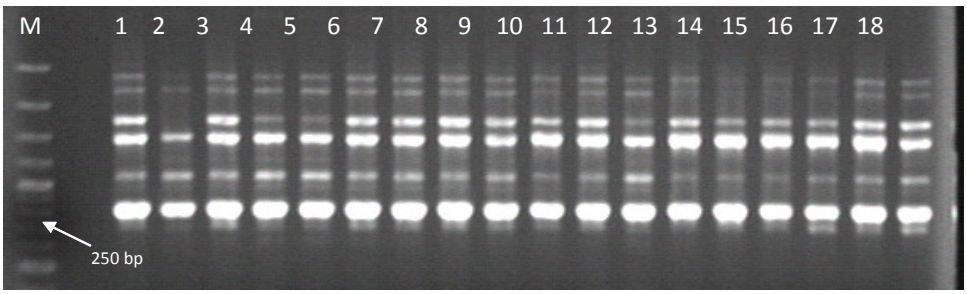

Figure 1. Polymorphic band patterns of different isolates for different agroecologies of Uganda i.e. M = 1 kb ladder (Fermentas Life Sciences).

Table 4. Gene diversity and number of polymorphic loci of Ugandan *Colletotrichum sublineolum* population based on Inter Simple Sequence Repeat polymorphism

District of origin	Number of polymorphic loci [b]	Average gene diversity ($) [a]
Arua	20	0.254
Masaka	29	0.264
Hoima	33	0.312
Nebbi	32	0.335
Tororo	30	0.274
Bushenyi	21	0.224
Apac	27	0.192
Sororti	18	0.324
Lira	26	0.235
Dokolo	23	0.228
Amuria	23	0.260
Kaberamaido	32	0.283

[a] average gene diversity computed according to Nei (Nei, 1987) and Tajima (Tajima, 1993)
[b] The number of polymorphic loci in comparison to the total number of 37 ISSR loci studied

Average gene diversity over all loci ranged from 0.192 to 0.335 showing high diversity within population rather than between populations (Table 4). The number of polymorphic loci were similar for the population studied (a locus is considered polymorphic if the most common allele has a frequency of less than 95% in all of the populations analysed) (Table 4).

Phylogenetic analysis. No significant clusters were found as demonstrated by bootstrap values less than 95% (Fig. 2). Nevertheless the data shows some trend in the clustering of isolates. In general, isolates, from a similar region for example Northern and North-Eastern Uganda which comprised the Northern moist farm lands tended to cluster together (Fig. 2). For example isolates marked lira, Dokolo, Kaberamaido all clusted together. A similar trend was observed for the isolates from other agroecologies separated by wide geographical distances clustered together for example the isolate Tr2 from Tororo clustered largely with isolates from Arua located more than 600 Km. This pattern suggests presence of local populations as supported by the AMOVA analysis in section 4:3:1.

Discussion

Genetic variability in *Colletotrichum sublineolum*

This study was to assess the genetic variability in *Colletotrichum sublineolum* population in Uganda. Determination of genetic or population structures provides insights into the degree of genetic diversity of a pathogen or any other organism and lays a foundation for its population genetic characterisation. DNA markers have provided a powerful tool for this type of study. In the last one and half decades, DNA markers have been used to quantify genetic variation (Balardin *et al.,* 1997), decipher spatial distribution (Milgroom and Lipari, 1995), and distinguish among forces that generate and maintain genetic variation for several agriculturally

Figure 2. Neighbour-Joining dendrogram of 124 *C. sublineolum* isolates derived from Inter-Simple sequence Repeats (ISSR). Branch length is proportional to the genetic distance which is indicated by the bar. Only bootstrap values above 50 are indicated.

important fungi. In this study, an estimate of Wright's population fixation ΦF_{ST} value was 0.08. In other fungal pathosystems, population differentiation indices within this range have been interpreted as indicative of moderate genetic differentiation between populations (Gagné, *et al.,*. 2001). The presence of a moderate population structure among the populations, suggests that the pathogen population in this study have been genetically isolated from each other. The populations deriving from the same original population have got to be reproductively and genetically isolated from each other to permit selection and fixation of unique alleles that will account for divergence and presence of genetic structures (Hartl and Clark, 1997). Uganda is located close to the secondary center of diversity of sorghum and millet. It is therefore not surprising that this study finds a genetically diverse population within the various agroecologies.

It is expected that in centers of diversity pathogens and their hosts have co-evolved and as such contain diverse population. Interestingly, the data also shows that all population have very high gene diversity on an average of 0.2. Gene diversity is a good tool for estimating genetic variability among organisms that reproduce asexually (Weir, 1996). Such data as found in this study suggest a highly variable clonal populations of *C. sublineolum.* Other studies have shown that *C. sublineolum* largely reproduce asexually and population structures may be found even within the same field (Rosewich *et al.,* 1998). On the basis of population structure, gene flow, and presence or absence of sexual phases, the potential evolutionary risk associated with fungal plant pathogens can be predicted (McDermont *et al.,* 1993). The presence of a population structure among Ugandan *C. sublineolum* from a management point of view suggests that the evolutionary responsiveness of this pathogen to selective disease control strategies may be moderate.

The population dynamics of a species is driven by interactions among mutation, migration, genetic drift, mating system, and selection, but it is rare to have sufficient empirical data to estimate values for all of these forces and to allow comparison of the relative magnitudes of these evolutionary forces. The data from phenetic analysis alludes to local populations of the pathogen as indicated by clusters of isolates from the same agro-ecologies. These data suggest limited gene flow or at least a strong effect of selection and perhaps genetic drift in the Uganda *C. sublineolum* population leading to population differentiation.

Taken together, these results imply that for long-term disease management, host disease resistance should form a core component of disease management due to moderate evolutionary responsiveness in *C. sublineolum.* Spatial characterisation of anthracnose epidemics could elucidate disease carry over. This information could help improve disease control in the tropics and elsewhere. Moreover, the presence of population structure suggests that there is a local population of *C. sublineolum* in Uganda. Thus disease management strategies should target deployment of resistant lines as first line of defense, and coupled with cultural practices to provide long term disease management.

Conclusion and recommendations

In the present study, genetic variability of *C.sublineolum* population in Uganda was

studied to gain insights of its evolutionary history and potential to evolve. This paper is based on the premise that population structure, gene flow, and study of presence or absence of sexual phases, can be used to explain the potential evolutionary risk associated with fungal plant pathogens. It is hoped that the results presented here shall be useful in the design and implementation of disease management strategies for sorghum anthracnose in the different agroecological zones of Uganda.

What is clear from this study, is the fact that *C. sublineolum* is a highly variable pathogen devastating sorghum production. In this study neutral genetic markers (ISSR) were used to investigate population differentiation. Neutral genetic markers have their major weakness of being selectively neutral and thus cannot detect effects of selection in populations. The use of selectable markers such as race differentials will provide insights on the impact of sorghum variety selection and also determine the different types of races in Uganda. There is thus a need to conduct further studies using race differentials. This will improve on management of sorghum anthracnose in Uganda in particular and Eastern Africa in general.

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