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Full Length Research Paper

Genetic homogeneity among Ugandan isolates of *Xanthomonas campestris* pv. *musacearum* revealed by randomly amplified polymorphic DNA analysis

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The Random Amplification of Polymorphic DNA (RAPD) analysis was used to detect the genetic diversity among Ugandan isolates of *Xanthomonas campestris* pv. *musacearum* (Xcm), the causal agent of banana Xanthomonas wilt (BXW) disease. Seven random primers were used because of their ability to amplify reproducible and reliable fingerprints generated between 6 - 12 amplicons each from the Xcm isolates obtained from central core of pseudostems, peduncles, fruit peelings, sap, nectar, insects' bodies and bacterial oozes. Regardless of the source and geographical origin, similar fingerprints were generated from the tested isolates. Using a similarity coefficient of 58%, the unweighted pair group method with arithmetic averaging (UPGMA) analysis did not reveal any significant differences in clustering, with exception of a single isolate that had unique fingerprints. Prior to the genetic analysis, all the isolates compared showed no significant difference (P = 0.92) with regard to incubation period for appearance of symptoms and the severity of symptoms in pathogenicity test. Thus, our data indicates that the population of Xcm in Uganda is clonal, that is, one uniform population being spread fast and efficiently, suggesting that there is a low likelihood of the current population to rapidly evolve, in the near future, into more virulent strains to overcome any resistance deployed.

Key words: Banana Xanthomonas wilt, DNA fingerprints, genetic diversity, *Xanthomonas campestris* pv. *musacearum.*

INTRODUCTION

Banana Xanthomonas wilt (BXW) disease is currently considered a major disease of banana across East and Central Africa because of its wide spread distribution and destructiveness (Eden-Green, 2004; Tushemereirwe et al., 2006; Biruma et al., 2007; Smith et al., 2008; Tripathi et al., 2009). The disease was first recognized as a significant production constraint on enset (*Ensete ventricosum)* and banana (*Musa* spp.) in the Ethiopian highlands four decades ago (Yirgou and Bradbury, 1968, 1974). However, its spread outside Ethiopia remained unknown, until 2001 when it was reported on banana in Uganda (Tushemereirwe et al., 2003, 2004). Since then, more outbreaks have been reported in eastern Democratic Republic of Congo (DRC) (Ndugo et al., 2006), Rwanda (Reeder et al., 2007), Tanzania, Kenya and Burundi (Carter et al., 2009). BXW spreads principally through contaminated cutting tools, infected planting materials or insects (Tushemereirwe et al., 2003; Eden-Green, 2004; Tinzaara et al., 2006). Diverse symptoms are displayed by BXW infected banana plants including progressive yellowing and wilting of leaves, premature and uneven ripening of fruits, rotting of bunches and shriveling and blackening of the male buds (Tushemereirwe et al., 2003; Tripathi et al., 2009). The

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Abbreviations: BXW, banana Xanthomonas wilt; **Xcm,** *Xanthomonas campestris* pv. *musacearum*

BXW infection causes absolute yield loss in all cultivated banana varieties and therefore, threatens the national food security and income of banana dependent households in the Eastern Africa region (Karamura et al., 2006; Smith et al., 2008; Tripathi et al., 2009).

The bacterium causing BXW disease was originally identified as *Xanthomonas campestris* pv. *musacearum* (Xcm) (Yirgou and Bradbury, 1974). However, recent studies using sequences of DNA *gyrase* sub unit B gene, substantiated by fatty acid methyl ester (FAME) and Repetitive polymerase chain reaction (Rep-PCR) profiling, showed that strains of Xcm from Uganda, Ethiopia, DRC and Rwanda belong to the same species of *Xanthomonas* with *X. vasicola* pv. *vasculorum* (Xvv) and *X. vasicola* pv. *holcicola* (Xvh) (Aritua et al., 2008). In addition, pathogenicity studies revealed that Xcm is able to induce a hypersensitive response in maize. Based on phylogenetic and biochemical analyses and pathogenicity studies, *X. vasicola* pv. *musacearum* (Xvm) was proposed as the new identity of the bacterium causing BXW (Aritua et al., 2008). However, more data on the pathogenicity characteristics of the *X. vasicola* pathovars are needed to support the new pathovar classification.

The current control measures for BXW are based on experiences with other closely related diseases like Moko disease caused by *Ralstonia solanacearum* (Thwaites et al., 2000). These include removal of male buds, disinfection of cutting tools, farmer-managed restriction of foreign planting materials and destruction of infected host plants (Tushemereirwe et al., 2006; Biruma et al., 2007; Tripathi et al., 2009). None of these control measures, however, has been found to be independently effective against BXW (Ssekiwoko et al., 2006; Welde et al., 2006). As with most plant bacterial diseases, resistant varieties would be the most cost effective and environmentally friendly method of managing the diseases. Development of disease-resistant varieties through conventional breeding requires resistant donor parents. Unfortunately, recent screening studies showed that none of the banana germplasm is resistant to the BXW (Ssekiwoko et al., 2006; Welde et al., 2006; Tripathi et al., 2008). Even if resistant germplasm sources are identified, conventional breeding of banana is difficult and lengthy process due to sterility of most cultivars coupled with long generation times. To circumvent these difficulties, transgenic technologies may provide a cost-effective alternative solution to the BXW pandemic (Tripathi et al., 2009).

The development and deployment of cultivars with durable resistance to BXW, whether through conventional breeding or using transgenic approach would, however, necessitate a detailed understanding of the genetic diversity in pathogen populations. The detection of variation in pathogen populations has traditionally relied upon use of phenotypic characteristics such as pathogenicity assays, morphological and biochemical tests. Since phenotypying is time-consuming and highly error- prone, several molecular techniques are used these days to examine pathogen diversity. To this end, a limited study by Aritua et al. (2007) on assessment of the genetic diversity within 25 isolates of Xcm populations from Uganda, DRC, Rwanda and Ethiopia using Rep-PCR, FAME analysis and the sequences of DNA *gyrase* sub unit B gene revealed that the current populations are homogenous. In the current study, we further examined the genetic diversity among 32 isolates of Xcm collected from different sources and geographic regions of Uganda using Random Amplified Polymorphic DNA (RAPD) analysis.

MATERIALS AND METHODS

Bacterial isolates

Thirty two isolates of Xcm were collected in the year 2005 from Ugandan banana growing districts affected by BXW (Table 1; Figure 1). More number of isolates (25) were collected from central Uganda, which is the main banana growing region followed by western region (3), eastern region (3) and northern region (1). In central Uganda, 12 isolates were collected from Mukono district as disease was first reported in Mukono in 2001 and then spread to other districts.

Xcm was isolated from the pseudostems, peduncles, fruit peelings, sap, nectar, insects bodies and bacterial oozes according to the procedures developed by Tripathi et al. (2007) and Mwangi et al. (2007). Pure cultures of each isolate were maintained on YTSA medium (1% yeast extract, 1% tryptone, 1% sucrose and 1.5% agar) at 4°C for short term storage and in glycerol (50% v/v in YTS) at -80°C for long term storage.

Pathogenicity test

To ensure that only pathogenic bacterial isolates were compared, a pathogenicity test was performed prior to the RAPD analysis. This was done by using artificial inoculation of bacterial suspension into the pseudostem of *in vitro* plantlets of the most susceptible banana cultivar 'Pisang awak' according to the protocol developed by Tripathi et al. (2008). Briefly, 10 plantlets were inoculated with 100 µl of bacterial suspension (10^8 cfu ml^{-1}) of each isolate. Control plantlets were injected similarly with sterile water. The inoculated plantlets were assessed every day for 6 weeks for appearance of BXW disease symptoms; typically chlorosis or necrosis and complete wilting of plants. The bacteria were re-isolated from symptomatic plants for identification.

RAPD analysis

Genomic DNA was extracted from Xcm isolates using the small-scale genomic DNA extraction protocol described by Mahuku (2004). DNA integrity and quality was checked by electrophoresis in 0.8% (w/v) agarose gels in 0.5X Tris-borate-EDTA and quantified by using spectrophotometer (Biomate, thermoscientific, USA). Each DNA sample was adjusted to a concentration of 10 ng μl^{-1} with sterile double-distilled water and stored at –20°C until use. RAPD analysis was performed using random primers (10-mer oligonucleotides with arbitrary sequences) (Margaret et al., 1996) synthesized at the University of Cape Town, Molecular and Cell Biology Synthetic DNA Laboratory. Initial optimisation was performed with 11 random primers and 3 bacterial isolates. Seven of the 11 primers (OPAM 1, OPAM 4, OPG 3, OPAG 20, OPAX 14, OPAW 11 and OPAH 11) that generated reproducible fingerprints were then used in the RAPD analysis. PCR amplifications were carried

Table 1. Summery of the origin of *X. campestris* pv. *musacearum* isolates used in this study.

Region	Districts	Number of isolates Per district	Number of isolates Per region
Central	Mukono	12	25
	Mpigi	6	
	Wakiso	3	
	Nakasongola	1	
	Kayunga	2	
	Masaka	1	
Eastern	Iganga	1	3
	Kamuli	1	
	Soroti	1	
Nothern	Lira	1	1
Western	Masindi	1	3
	Bushenyi	1	
	Kibale	1	
Total		**32**	**32**

out in a 12.5 µl reaction volume that contained 1.25 µl of 10X PCR buffer (PROMEGA, Madison, WI, USA), 2 µl $MgCl_2$ (2.5 mM) (PROMEGA, Madison, WI, USA), 1.25 µl of each primer (100 µM), 1.25 mM dNTPs, 20 ng of DNA and 0.5 units of Taq polymerase (PROMEGA, Madison, WI, USA). A negative control containing all components except the DNA extract was included in each PCR reaction. PCRs were performed in a 96-well Eppendorf thermo-cycler (EPPENDORF North America, USA) using the following conditions: pre-heated at 94^0C for 4 min and processed through 40 cycles consisting of 30 s of denaturation at 94^0C, 45 s of annealing at 37^0C and 1 min of extension at 72^0C, followed by final extension at 72^0C for 7 min. PCR for each DNA/primer combination was repeated at least twice to ensure reproducibility. The entire PCR amplification product was run on 1.2% agarose gel in 0.5X Tris-borate-EDTA for at least 120 min at 90 V. To determine molecular sizes of fragments generated for a comparative analysis, a 100 bp DNA ladder (ROCHE Diagnostics, Switzerland) was concurrently run in each gel. Gel images were captured using Vilber Lourmat gel documentation system (Vilber Lourmat Deuschland GmbH, Germany).

Data analysis

Bands generated by the RAPD analysis were visually scored as present (1) or absent (0) to generate binary data. Gel fingerprints were considered to be similar when all visible DNA bands had the same migration distance. Only clear unambiguous and reproducible bands were recorded (Trebaol et al., 2001). Cluster analysis and drawing of dendrogram was performed using unweighted pair group method with arithmetic averages (UPGMA), with NTSYS soft-ware (Version 2.02).

RESULTS

Pathogenicity test

All plantlets inoculated with bacterial suspension of the Xcm isolates developed symptoms typical of BXW (leaf chlorosis or necrosis), on average of 11 days after inoculation (Figure 2). All of the plants wilted completely in 24 - 28 days after inoculation, whereas control plantlets inoculated with sterile water remained healthy. There was no significant difference (P = 0.92) observed among the different isolates for wilting incidence and severity of symptoms. Bacteria were recovered from all wilted plants and identified as Xcm based on appearance of typical circular yellow mucoid colonies on semi-selective medium (Tripathi et al., 2007).

RAPD analysis

Initially, 11 RAPD primers were tested with 3 isolates of Xcm in order to select a set of RAPD primers which produce reproducible and reliable fingerprints for the further study. Seven out of the 11 primers were subsequently selected for the assessment of the genetic diversity within the Xcm isolates because they produced reproducible fingerprints. The primers differed in the number of amplified fragments generated; ranging from 6 for OPAG 20 as the least and 12 for OPAX 14 (Figure 3). Each primer, however, generated similar RAPD fingerprints for Xcm isolates in two separate PCR amplification reactions, irrespective of geographic origin or source of isolation (infected plant parts or an insect), except for isolate Wkk (Figure 3). The reason remains unknown since Wkk was obtained from Wakiso district in central Uganda with few other isolates and it was pathogenically similar to other isolates. All 32 Xcm isolates amplified with all the seven chosen primers.

To further examine their relatedness, RAPD fingerprints for 32 isolates were combined to construct a dendrogram based on the similarity in the polymorphisms of the DNA fragments generated. Similarly, the UPGMA

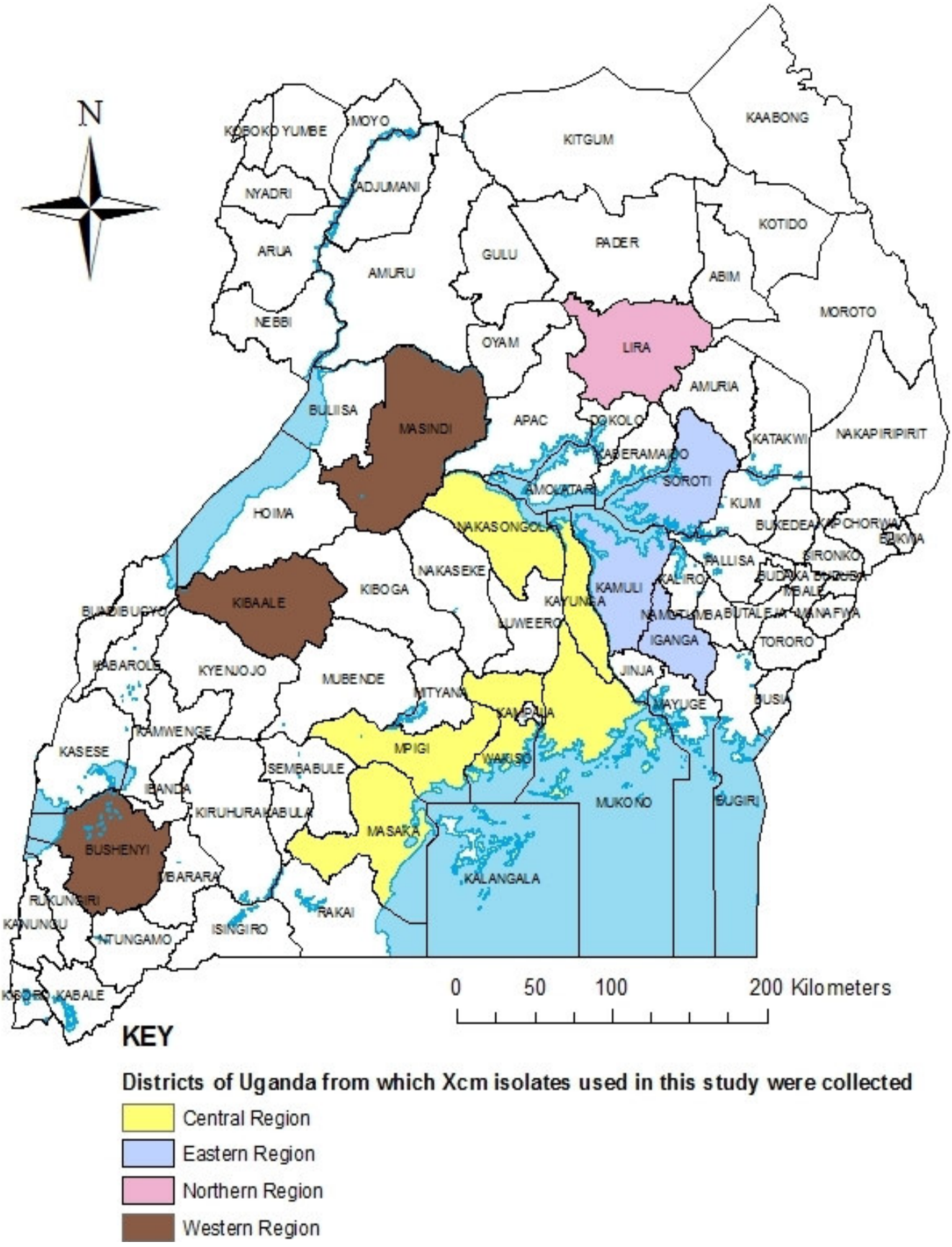

Figure 1. Map showing origin of *X. campestris* pv. *musacearum* isolates used in this study.

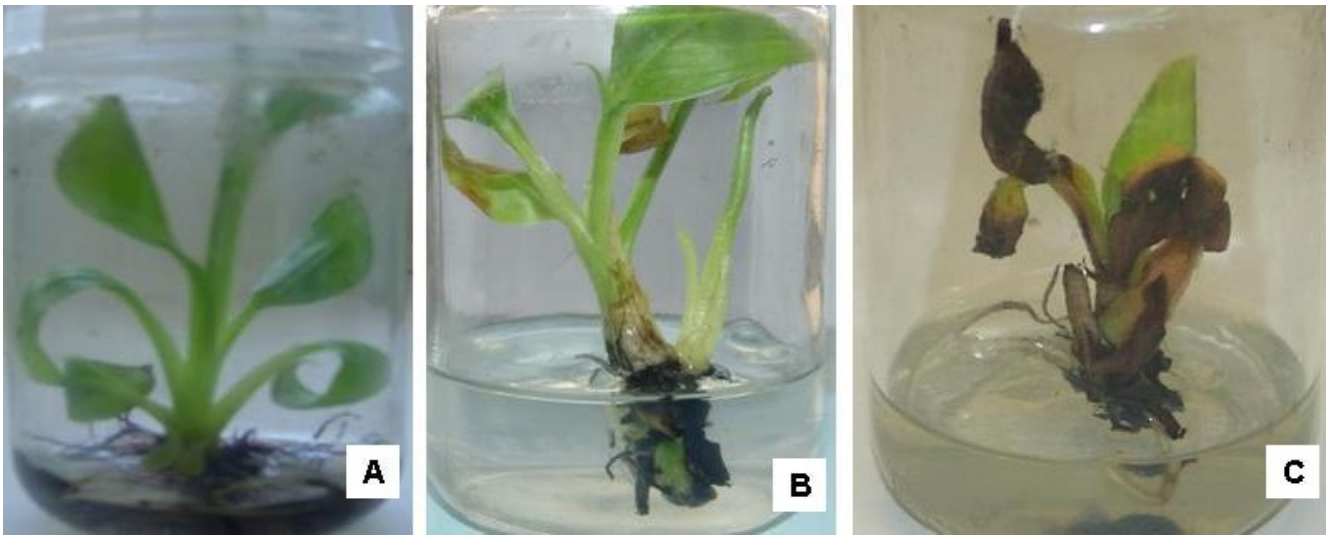

Figure 2. Banana plantlets cultivar 'Pisang awak' showing symptoms of Xanthomonas wilt disease after inoculation with *Xanthomonas campestris* pv. *musacearum* using *in vitro* screening method; A) asymptomatic control plantlet inoculated with water, B) plantlet showing initial symptoms of BXW, 12 days after inoculation, and C) plantlet showing wilting 6 weeks after inoculation.

analysis generated random clusters regardless of the geographical origin of the isolates. For example, isolate 036/Lra/05 (from Lira district in northern Uganda) clustered together with isolate 023/Kay/05 (from Kayunga district in central Uganda). Isolate Ib3/02/09/05 (from Mpigi district in cental Uganda) clustered with isolate Sor1 (from Soroti district in eastern Uganda) (Figure 4). At 58% similarity coefficient level, isolate Wkk that had unique gel fingerprints clustered alone while the rest of isolates randomly clustered in one cluster.

DISCUSSION

PCR-based approaches have been used to study genetic diversity of phytopathogenic bacteria and generate evidence of their ecological distribution, dispersal and evolution (Leung et al., 1993; Adhikari et al., 1999; Kumar et al., 2004). In this study, the genetic diversity within Ugandan Xcm population was assessed using RAPD analysis. In absence of the complete sequence information about the genome of the pathogen, RAPD was the ideal technique to use since it scans for sequence variation throughout the whole genome. Overall, limited genetic variability was observed among most of the assessed Xcm populations in Uganda, with the exception of isolate Wkk that appeared to differ from the rest of the isolates, despite being pathogenic on banana (Figures 3 and 4). This finding together with earlier reports of high similarity in pathogenicity within Ugandan isolates (Tripathi et al., 2008) and high genetic homogeneity among isolates from Uganda, DRC, Ethiopia and Rwanda (Aritua et al., 2007) confirms the existence of limited genetic diversity within the current populations of Xcm in East and Central Africa. Some of the Xcm isolates used in the current RAPD analysis were previously used in the preliminary genetic diversity study conducted by Aritua et al. (2007). Thus, the large number of geographically widely distributed isolates used in current study, and the choice of a technique that scans for diversity in the whole genome gives strong credence to the existence of high genetic homogeneity among Xcm populations.

High genetic similarity has also been reported in other bacterial pathogens. Kumar et al. (2004) found out that strains of *Ralstonia* causing bacterial wilt of ginger and other hosts exhibit a high level of genotypic similarity. They hypothesized that lineages of single virulent strain seem to be transmitted from one location to another along with rhizomes, which act as a protected carrier. Although it remains speculative, this appears to be the case with Xcm. Thus, it is possible that the clonal propagation of bananas, largely by the use of suckers as planting material continues to spread the same stains of Xcm in asymptomatic planting materials, in addition to the unintentionally contaminated cutting tools or insect vectors, as evidenced by prevalence of identical fingerprints found in this study. We suspect that the same strain that was introduced in Mukono district in central Uganda in 2001 (Tushemereirwe et al., 2003, 2004) has spread to other parts of Uganda, perhaps including other parts of East and Central Africa, in the same planting material.

The East African highland region is widely recognised as a secondary center of banana diversity (Karamura, 1998). Accordingly, the BXW is expected to be subjected to varying selective pressures as they encounter the various genetic nature of the host (banana). This should have resulted into variability as they selectively adapt to

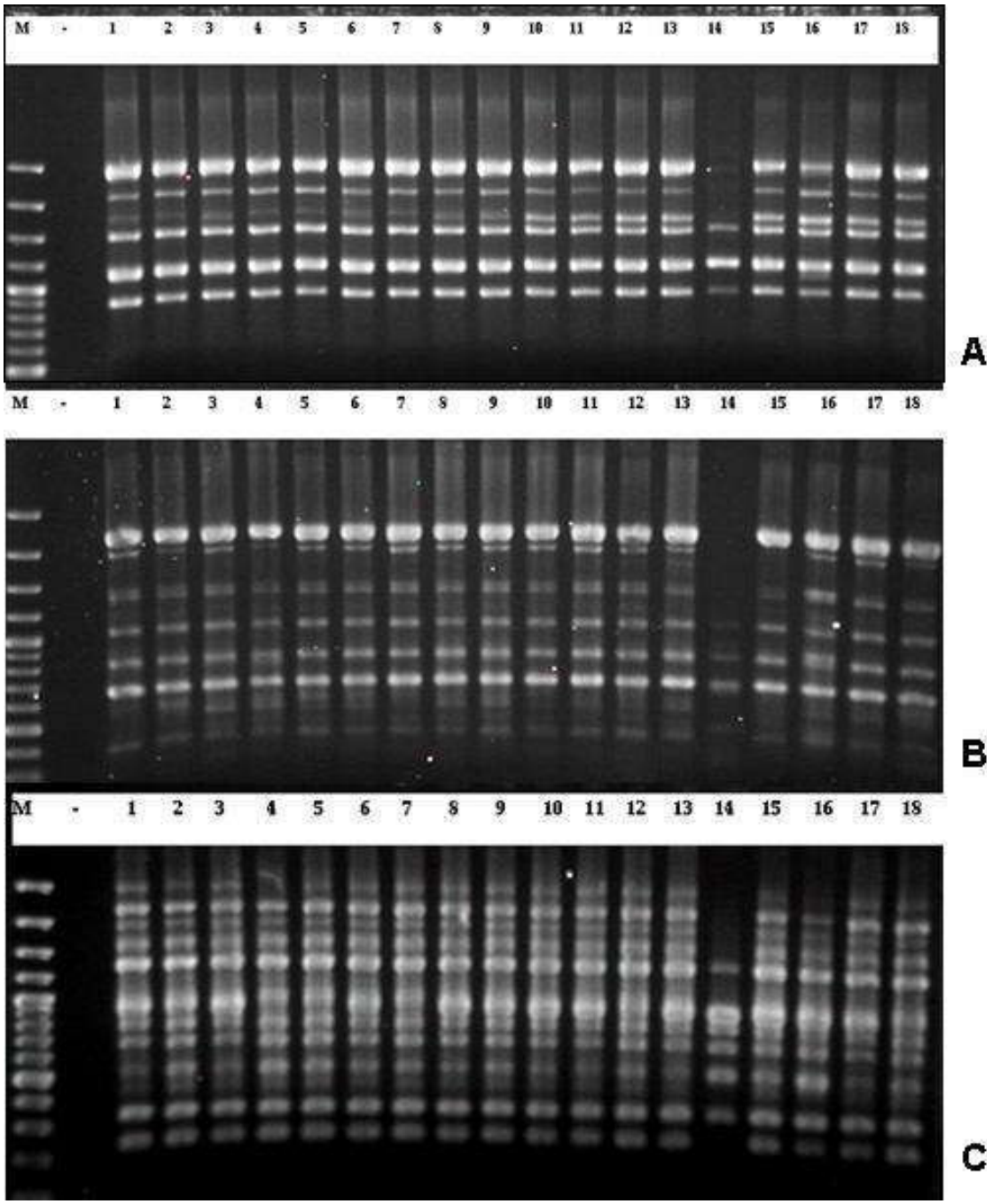

Figure 3. Representative electrophoresis gel picture showing RAPD-PCR amplification of genomic DNA from 18 isolates of *X. campestris* pv. *musacearum* using RAPD primers OPAG 20 (A), OPG 3 (B) and OPAX 14 (C). Lanes: M,100-bp ladder marker DNA; ., Negative control; 1 to 18;- N1/03/08/05, O4/30/08/05, 005/wks/05, P10/01/02/05, S2/31/08/05, S1/27/19/05, O7/30/08/05, 046/Bush/05, O3/29/08/05, 043/Msd/05, OZ1/03/08/05, O8/27/08/05, 006/Wks/05, Wkk, Sor 1, Mak, P3/01/03/05, S7/16/08/05.

the various pressures. Therefore, failure to change since the first introduction suggests that the Xcm population in Uganda has not been exposed to severe selection pressure, confirming the earlier argumentation by Aritua et al. (2007) that selective pressure from the host plant, the vector(s) or the environmental conditions seem to have played insignificant role in selecting different populations of Xcm. Alternatively, the 7 years since the first identification of Xcm in Uganda has not been long enough to generate a strong selection pressure from the banana host to cause a significant change in the pathogen. The reason for the uniqueness of isolate Wkk, however, still

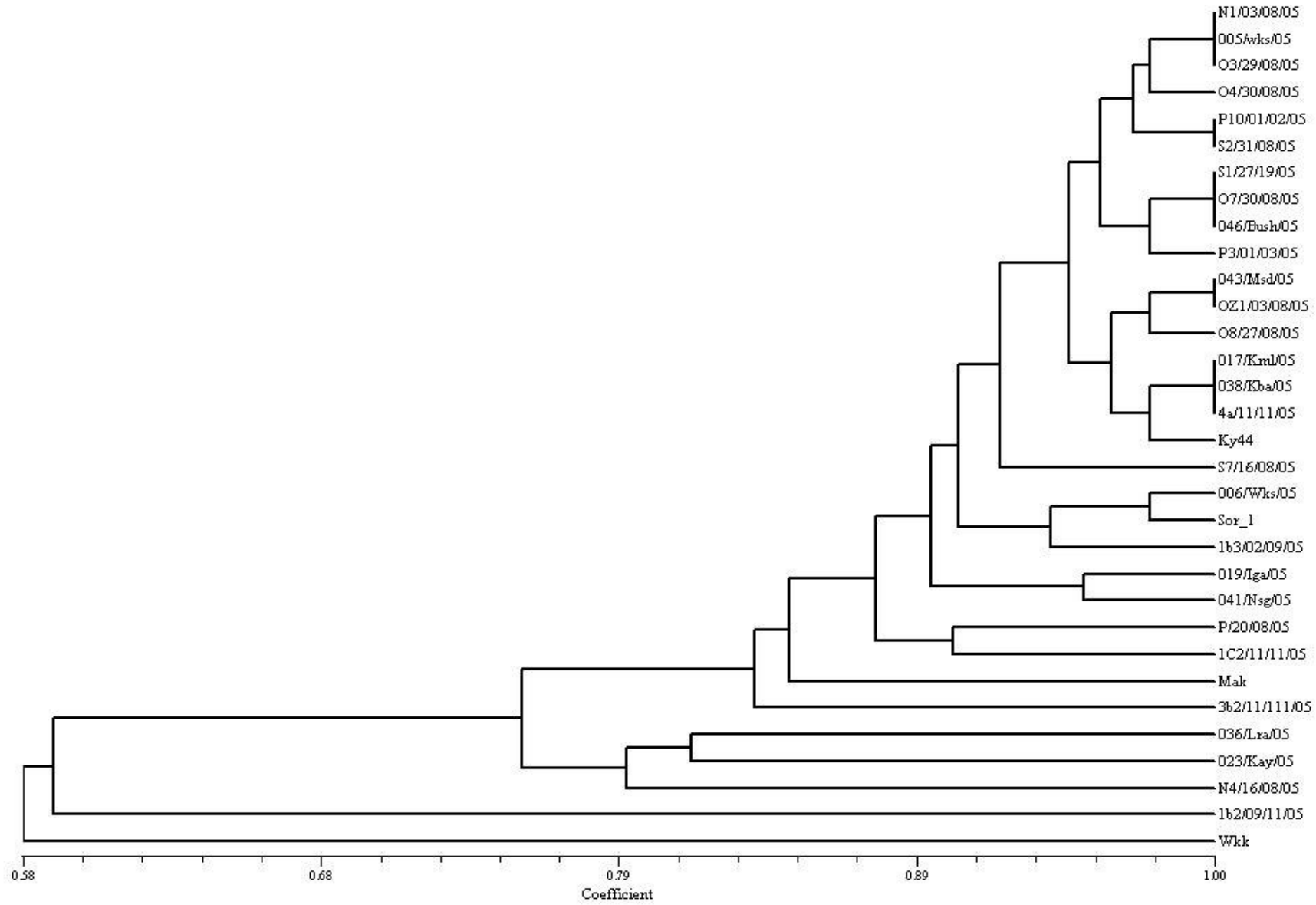

Figure 4. Unweighted pair group method, arithmetic average (UPGMA) dendrogram constructed from randomly amplified polymorphic DNA of *Xanthomonas campestris* pv. *musacearum* isolates collected from various districts in Uganda.

remains to be established. One can only speculate that it is a result of chromosomal changes such as insertion, deletion, or inversion in the amplified regions (Foolad et al., 1995).

The random clustering of isolates from different geographical regions could be due to the continuity of the main banana growing regions in Uganda. It is possible that a single isolate was introduced in Uganda and simultaneously spread in all parts. Moreover, the farmer practice of obtaining plant materials from near and far, without taking precautions to avoid carrying contaminated suckers and moving that to another place over a long distance could have perpetuated the same isolate.

In conclusion, our study indicates that the population of Xcm causing BXW in Uganda is clonal, that is, one uniform population being spread fast and efficiently. The results suggest that there is a low likelihood of the current population to rapidly evolve, in the nearest future, into more virulent strains to overcome any resistance deployed. Farmers can, therefore, go on with use of the recommended BXW management practices that have so far exerted no or limited selection pressure on Xcm population in Uganda. Additionally, the high genetic homogeneity indicates that development and wide use of a single resistant variety will effectively control the disease.

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