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Phenotypic and genotypic diversity of *Xanthomonas axonopodis* pv. manihotis causing bacterial blight disease of cassava in Kenya

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ABSTRACT

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is the most important bacterial disease of cassava. There is no information available on the morphological and genetic variability of *Xam* isolates from Kenya. The aim of this study therefore, was to determine the diversity of *Xam* isolates from different cassava growing regions of Kenya using phenotypic characteristics and repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR). Thirty three isolates were recovered from infected cassava leaf samples collected from farmers' fields in cassava growing regions. The dendrogram generated from analysis of phenotypic characteristics of the isolates produced two major clusters at 75% similarity level. Analysis of 19 isolates with repetitive extragenic palindromic (rep) primers yielded characteristic fingerprint pattern with bands ranging between 400 and 2000 bp in size and their numbers ranged from 1 to 6 bands per isolate. Cluster analysis using unweighted pair group method with arithmetic averages (UPGMA) did not reveal any significant differences in clustering and relationship to the geographical origin, with exception of a single isolate that had unique fingerprints. These findings indicate that *Xam* population in Kenya evolved from the same origin and is a uniform population, and this may prove useful in future breeding programmes.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important source of food and income to more than 300 million people in sub-Saharan Africa. Its tuberous roots accumulate starch (approximately 30 - 60% dry matter) and it is considered the second source of starch globally, after maize [1]. In Kenya, cassava is the third most important food crop and its tuberous roots provide over a quarter of the daily calorie consumption. The leaves are consumed as a vegetable, providing an important source of proteins, vitamins and micronutrients. Cassava is drought tolerant and can be grown in marginal soils, typical of low-income, small-scale farmers, with minimum inputs. However, production of cassava is limited by viral, bacterial, fungal, phytoplasmas and insect-pest attacks on tuberous roots and plants. The most important disease that can cause high losses to subsistence farmers in Kenya is cassava bacterial blight (CBB). Cassava bacterial blight (CBB) disease caused by *Xanthomonas axopodis* pv. *manihotis* (*Xam*) is a major disease of cassava across sub-Saharan Africa and South America. It is a vascular disease that reduces the production of cassava up to 100% since it affects both planting material and yields [2, 3]. Diverse symptoms are displayed by CBB infected cassava plants including wilting of leaves, blighting, formation of angular leaf lesions, stem cankers, stem and leaf exudates production and, dieback of stems. Infection of CBB commences with the multiplication of *Xam* in the leaves around the stomata and eventually enters into vascular system through the stomatal pores [4]. The entry of *Xam* into the vascular system triggers defense-response, which include suberin deposition, lignin deposition, accumulation of phenolic compounds, and occlusion of vessels. The degree of the impact of CBB varies depending on the population of pathogens, their pathogenicity, and genotypes of cassava [5]. The control measures for CBB include use of clean propagative cuttings, crop rotation, planting towards the end of the rainy season and removal of infected material. However, none of these control measures have been found effective against CBB. These methods are aimed at reducing bacterial populations in the field [6].

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As with most plant bacterial diseases, the use of resistant varieties would be the most effective method of managing CBB. However, development of disease-resistant varieties through conventional breeding requires resistant donor parents. The development and deployment of cultivars with durable resistance to CBB necessitates a detailed understanding of the diversity of pathogen populations. Variation of pathogen populations can relate to differences in pathogenicity and/or virulence of isolates of the same pathogen [7], and such differences can be attributed to differences in genetic makeup of the bacterial isolates. Therefore, for effective control of CBB, understanding the genetic variation of pathogen populations in different regions is important for breeding because plants need to have resistance against all pathogenic strains. In addition, in order to develop efficient disease management strategies, the genetic diversity of the pathogens population under the climatic conditions studied must be known since genetic diversity of *Xam* has been reported to be directly correlated with environmental conditions [8]. If the genetic variation of the pathogen is not considered in the different cassava growing regions, it may lead to an increase or decrease in the virulence which might in turn lead to susceptibility in cultivars that were previously resistant to the pathogen [9].

Phenotypic characterization is an important first step in the assessment of diversity of pathogenic bacteria; however it is time consuming and is highly error prone due to morphological plasticity, which is influenced by environmental conditions. Therefore, pathogen diversity can be augmented by combining both genotypic differences between strains and phenotypic analyses. A number of PCR based methods have been used for identification and genetic characterization of *Xam* isolates including use of random amplified polymorphic DNA (RAPDs) [10], restriction fragment length polymorphism (RFLPs) [11], amplified fragment length polymorphisms (AFLP) [12] and repetitive extragenic palindromic-polymerase chain reaction (rep-PCR). Repetitive extragenic palindromic PCR technique has been shown to be simple and fast and thus useful for studying plant pathogen population structures [13, 14]. It has been used successfully for characterizing *xanthomonads* and *pseudomonads* [13, 14]. Therefore, rep-PCR is strongly recommended for genetic characterization of pathogenic bacteria because it is more precise in terms of identification and evaluation of diversity [15].

To date, no information is available regarding the phenotypic or genetic variability of *Xam* populations from cassava growing regions in Kenya. Therefore, the objectives of this study were to identify phenotypic and genetic variability of *Xam* isolates collected from different cassava growing regions of Kenya.

2. MATERIALS AND METHODS

2.1 Collection of infected cassava samples

Leaf samples were collected from cassava plants showing bacterial blight symptoms from five cassava growing regions (Western, Coastal, Nyanza, Central and Eastern) of Kenya (Figure 1). A total of 33 cassava leaf samples were randomly collected from farmers fields (Table 1). More samples were collected from Western (18) and Coastal regions, (8) which are the main cassava growing regions followed by Nyanza (5), Eastern (1) and Central (1). The samples were collected in sterile polythene bags containing silica gel, transferred to the laboratory at the Department of Biochemistry, University of Nairobi and stored at room temperature for subsequent *Xam* isolation and characterization.

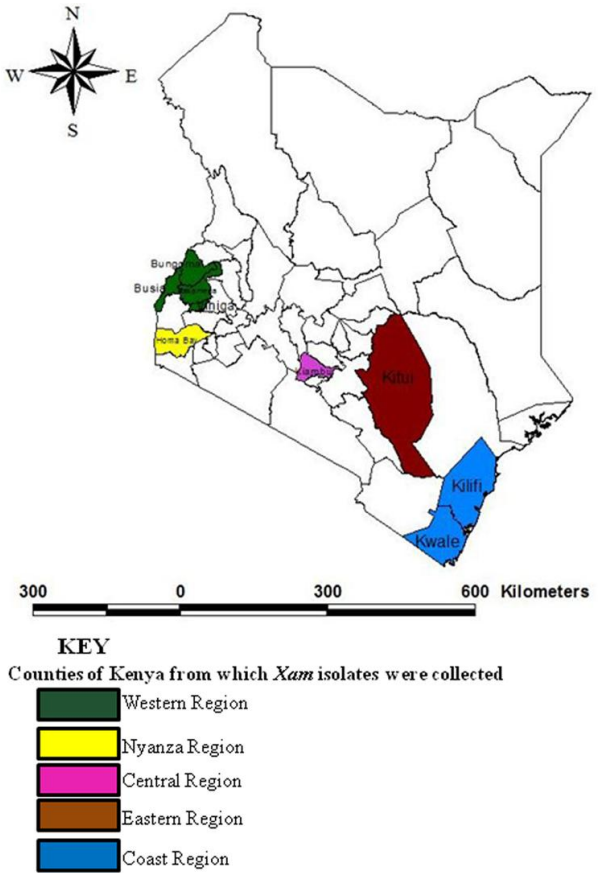

Fig. 1: Map of Kenya showing Counties from which *Xam* isolates used in this study were collected.

Table 1: Summary of the origin of *X. axonopodis* pv. *manihotis* isolates used in this study

Region	County	Number of isolates per County and their ID code	Number of isolates per region
Western	Kakamega	7 (Xam1-W, Xam2-W, Xam3-W, Xam4-W, Xam5-W, Xam6-W and Xam18-W)	18
	Vihiga	5 (Xam7-W, Xam8-W, Xam9-W, Xam10-W and Xam11-W)	
	Bungoma	2 (Xam12-W and Xam13-W)	
	Busia	4 (Xam14-W, Xam15-W, Xam16-W and Xam17-W)	
Eastern	Kitui	1 (Xam19-E)	1
Nyanza	Homabay	4 (Xam20-N, Xam21-N, Xam22-N, Xam23-N and Xam24-N)	5
Central	Kiambu	1 (Xam25-Ce)	1
Coast	Kilifi	6 (Xam26-C, Xam27-C, Xam28-C, Xam29-C, Xam30-C and Xam31-C)	8
	Kwale	2 (Xam32-C and Xam33-C)	

2.2 Isolation of bacteria

Isolation of the pathogen was done as described by Verdier *et al.* [16] and Ogunjubi and Fagade [17]. Leaf tissues were macerated into small pieces of about 2 mm^2 and transferred into sterile 15 ml falcon tubes. About 5 ml YPG-CC (yeast extract (1%), peptone (1%), glucose (1%), agar, cephalexin (50 mg l^{-1}) and cycloheximide (150 mg l^{-1}), pH 7.0.) liquid selective media was added and incubated for 48 hours at 28 °C. The bacterium suspension was diluted serially to 10^{-3}, and aliquots of 0.1 ml of each of the 10-fold dilution samples transferred onto YPGA-CC medium (yeast extract (1%), peptone (1%), glucose (1%), agar (1.5%), pH 7.0) supplemented with cephalexin (50 mg l^{-1}) and cycloheximide (150 mg l^{-1}).

The Petri dishes were incubated at 28 °C for 48 hours. Single colonies were selected and purified by re-streaking on YPGA-CC, grown at 28 °C for 48 hours and kept at -20 °C for storage in 30% glycerol.

2.3 Identification of *Xam* isolates

Identification of bacterial isolates was carried out using both the Gram staining reaction and 3% KOH test. Gram staining reaction was done as described by Bradbury [18]. Gram staining reaction was confirmed by performing 3% KOH test. Pure bacterial colony was taken with a toothpick and vigorously stirred in one drop of 3% KOH solution.

After mixing, a tooth pick was raised a few centimetres from a glass slide to observe a strand of viscid mucous material as described by Gregersen [19]. If strands of viscid material were observed, the bacterial isolates were regarded as Gram-negative. Lack of strands of viscid material was recorded as Gram-positive.

2.4 Phenotypic characterization of *Xam* isolates

Bacterial suspensions of each isolate were streaked onto plates of YPGA-CC selective media. The plates were incubated at 28 °C for 48 hours followed by morphological characterization. All the isolates were observed on YPGA-CC plates for colony color, form, elevation, size, surface, edges, structures, growth rate of colonies and colony texture as described by Bradbury [20] and Fahy *et al*. [21].

2.5 Molecular characterization of *Xam* isolates

Out of the 26 *Xam* isolates recovered, 19 were selected based on hierarchical cluster analysis from morphological characterization for further genetic diversity studies.

2.5.1 DNA isolation

Extraction of genomic DNA from cultures of bacterial isolates was performed using DNeasy® mini kit (Qiagen, 2013) following manufacturer's instructions. The DNA was resuspended in sterile double distilled water. DNA integrity and quality were checked by agarose gel electrophoresis in 1X Tris-acetate-EDTA (TAE) and quantified using a spectrophotometer (Beck Man Coulter UV/VIS, USA)

2.5.2 Detection of Xam using PCR

The DNA extracted from cultures of bacterial isolates was subjected to PCR analysis using *Xam*-specific primers XV (5'-TTC-GGC-AAC-GGC-AGT-GAC-CAC-C-3') and XK (5'-TCA-ATC-GGA-GAT-TAC-CTG-AGG-G-3') in a MJ Mini™ personal Thermal Cycler (BIO-RAD, Singapore). PCR reactions were performed in a total volume of 20 µl thin-walled Accupower™ PCR tube (USA Bioneer, Inc.) using 1 µl of 1.5 mM $MgCl_2$, 4 µl of buffer 5X, 0.5 µl of 0.5 mM dNTPs, 0.5 µl of 10 µM of each primer, 0.5 µl of 1.25 units of *Taq* polymerase, 2 µl of 25 ng/µl of DNA and 11.5 µl of sterile double-distilled water. The PCR cycling conditions were an initial denaturation at 95 ºC for 2 minutes followed by 30 cycles of 94 ºC for 30 seconds (denaturation), 55 ºC of 1 minute (annealing) and 72 ºC for 1 minute (extension) and a final extension of 5 minutes. The PCR products were electrophoresed on a 1% (w/v) agarose gel in 1X TAE buffer containing 0.5µg/ ml of Gel Red and visualized in a UV transilluminator.

2.5.3 Repetitive elements based PCR (rep-PCR)

A total of 19 *Xam* isolates were analyzed. Amplification reactions were performed using primer pair: REP1R-2 (5′-IIIICGICGICATCIGGC -3′) and REP2-2 (5'–ICGICTTATCIGGCCTAC -3') as described by Louws *et al*. [22]. PCR amplifications were carried out in a 25 µl reaction volume consisting of 4 µl of 10X PCR buffer, 0.5 µl of each primer, 0.2 µl of *Taq* DNA polymerase (Qiagen), 0.5 µl of dNTPs, 1 µl of $MgCl_2$, 16.3 µl of double-distilled water and 2 µl (50 ng/µl) of bacterial DNA. A non-template reaction was included as a negative control. PCR reactions were performed in a MJ Mini™ personal Thermal Cycler (BIO-RAD, Singapore) using the following conditions: initial denaturation at 95 ºC for 7 minutes, followed by 30 cycles of 94 °C for 1 minute (denaturation), 44 °C for 1 minute (annealing) and 65 ºC for 8 minutes (extension) with a final extension at 65 ºC for 15 minutes. The PCR products were electrophoresed on a 1.5 % (w/v) agarose gel in 1X TAE buffer containing 0.5µg/ ml of GelRed and visualized in a UV transilluminator. All PCR reactions were repeated at least three times for each sample to verify the consistency of the patterns. Genomic fingerprint comparisons among *Xam* isolates were performed by measurement of band sizes. Only clear and reproducible bands were scored for each *Xam* isolate. Bands generated by the rep-PCR analysis were converted into a two-dimensional binary matrix (1, presence; and 0, absence of a band) using Jaccard (J) coefficient, which do not consider the negative similarities.

The matrix was analyzed by Popgene package. Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of *Xam* isolates

A total of 26 bacterial isolates were recovered from 33 cassava infected samples. The bacterial isolates produced large mucoidal colonies on YPGA-CC selective media (Figure 2). Biochemical tests of the bacterial isolates indicated that all the isolates were Gram-negative and produced mucoidal thread-like rods in 3% KOH (Figure 2).

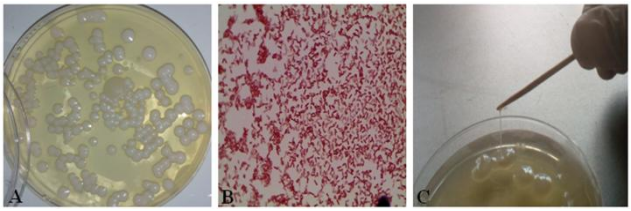

Fig. 2: Colonies of *Xam* isolated from infected leaf samples and results of biochemical tests. (A) colony of *Xam* on YPGA-CC; (B) mucous thread produced by Gram-negative bacteria on 3% KOH; and (C) microscopic view of Gram stained *Xam* isolate.

3.2 Phenotypic characterization

Colonies of cultured *Xam* isolates appeared after 48 hours on YPGA-CC selective media. The colonies of the isolates were white/cream/yellow, mucoid and circular/round on YPGA-CC (Figure 2). Colony color varied from white to cream in all the isolates except one isolate (Xam12-W) that was yellow (Figure 3). These observations confirm reports by Verdier *et al*. [23] who found that *Xam* colonies appear creamy and whitish in color and form distinct isolated colonies. In addition, Trujillo *et al*. [24] reported that *Xam* cultured on selective media is distinguished by white and cream colonies. Reports of variations in color, shape, elevation and contour (margin) among *Xam* strains have also been documented in previous studies [25, 26, 27].

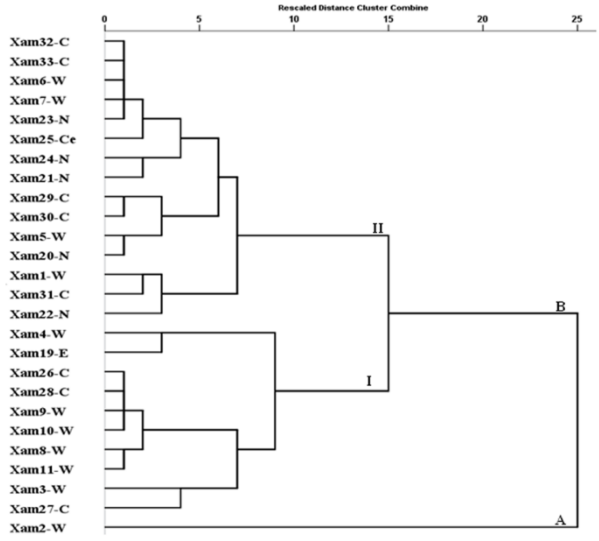

Fig. 3: Colonies of *Xam* after 48 hours of culture on YPGA-CC selective media showing the distinguishing features of *Xam*. (A) Xam1-W; (B) Xam12-W; (C) Xam21-N and (D) Xam25-C.

Phylogenetic analysis of the 26 *Xam* isolates resulted in two major clusters A and B. Cluster A contained only one isolate, Xam 2-W, obtained from western Kenya. Cluster B was further sub-divided into 2 sub-clusters. Sub-cluster I comprised ten isolates (Xam4-W, Xam19-E, Xam26-C, Xam28-C, Xam9-W, Xam10-W, Xam8-W, Xam11-W, Xam3-W and Xam27-C) (Figure 4) obtained from Coast, eastern and western regions of Kenya. Isolates in this sub-cluster were characterized by small, white, circular, smooth, mucoid translucent and elevated colonies. Sub-cluster II comprised of fifteen isolates (Xam22-N, Xam31-C, Xam1-W, Xam8-W, Xam5-W, Xam30-C, Xam29-C, Xam21-N, Xam24-N, Xam25-C, Xam23-N, Xam7-W, Xam33-C and Xam32-C) (Figure 4) obtained from Coast, Nyanza and western regions. Isolates in this sub-cluster were characterized by small, white, circular, smooth, moist and translucent colonies. The *Xam* isolates from the different geographical regions were randomly clustered. Differences in morphological characteristics have also been reported by McGuire and Jones [25] and Willems *et al*. [27].

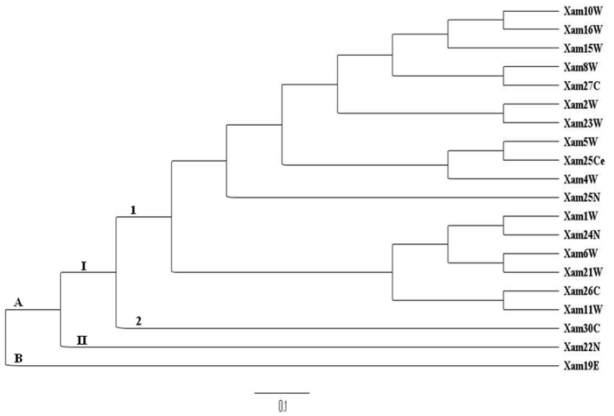

Fig. 4: Dendrogram of phenotypic diversity of 26 *Xam* isolates generated using morphological characteristics.

3.3 PCR analysis of *Xam* isolates

To confirm the microbiological and biochemical identification, 19 Kenyan Xam isolates were further tested by PCR. The PCR amplification of total genomic DNA was based on Xam-specific primers XK-XV. The primer pair XV-XK successfully amplified the expected 898 bp fragment of the bacterium DNA of all the 19 isolates.

3.4 Genetic diversity of *Xam* isolates by rep-PCR genomic fingerprinting

PCR-based approaches have been used to study genetic diversity of phytopathogenic bacteria and generate evidence of their ecological distribution and evolution [28, 29]. In this study, the genetic diversity within Kenyan *Xam* population was assessed using rep-PCR method.

All isolates produced characteristic fingerprint pattern with bands ranging between 300 and 3000 bp and their numbers ranged from 1 to 6 bands per isolate (Figure 5). The same fingerprints were observed when the PCR was repeated at least three times, demonstrating the reproducibility of this technique for

genetic studies of *Xam*. Some minor light amplification bands were also not reproducible, suggesting non-specific binding and such bands were not taken into account. This is in agreement with previous studies by Janssen *et al*. [30] and Restrepo *et al*. [32] who confirmed the usefulness of rep-PCR in characterizing bacterial populations and its applicability to the study of *Xam* populations.

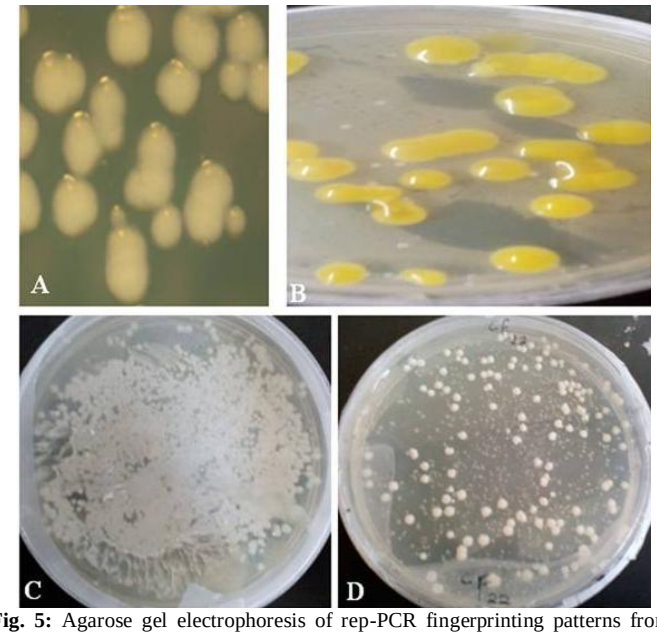

Fig. 5: Agarose gel electrophoresis of rep-PCR fingerprinting patterns from genomic DNA of *Xam* isolates obtained from infected cassava. Lanes M represent 1 kb molecular weight marker (Fermentas); Lanes 1 (Xam1-W), 2 (Xam24-N), 3 (Xam19-E), 4 (Xam26-C), 5 (Xam8-W), 6 (Xam6-W), 7 (Xam2-W),8 (Xam22-N), 9 (Xam27-C), 10 (Xam30-C), 11 (Xam20-N), 12 (Xam16-W), 13 (Xam16-W), 14 (Xam5-W), 15 (Xam21-N), 16 (Xam23-N), 17 (Xam4-W), 18 (Xam11-W) and 19 (Xam25-Ce) represent *Xam* isolates from Western (W), Nyanza (N), Coast (C) , Eastern (E) and Central (Ce) regions of Kenya.

Majority of isolates revealed complex banding pattern, with only four isolates exhibiting less than four bands. Isolates Xam19-E and Xam24-N generated a low complexity pattern with one and three bands, respectively. Although a lower number of clearly scorable bands were observed in some isolates (Figure 5), the three repeats of rep-PCR amplifications revealed that the absent bands are reproducible. The findings from the present study are in agreement with reports by Restrepo *et al*. [33] demonstrating the high discriminatory power of rep-PCR when characterizing *Xam* populations with low levels of genetic diversity in Colombia.

To examine the relatedness of Kenyan *Xam* populations, rep-PCR fingerprints for selected 19 isolates were used to construct a dendrogram based on the similarity in the polymorphisms of the DNA fragments generated. Overall, limited genetic variability was observed among most of the assessed *Xam* populations in Kenya, with the exception of isolate Xam-19E that appeared to differ from the rest of the isolates (Figure 6). At 10% similarity coefficient level, isolate Xam-19E that had unique gel fingerprints (only single band) clustered alone while the rest of isolates randomly clustered in one cluster. These finding confirms the existence of limited genetic diversity within the current populations of *Xam* in Kenya. The UPGMA analysis generated random clusters regardless of the geographical origin of the isolates (Figure 4).

For example, isolate Xam-11W from Vihiga County in Western region clustered together with isolate Xam-26C from Kilifi County in coastal Kenya. Isolate Xam-5W (from Kakamega County in western Kenya) clustered with isolate Xam-25Ce (from Kiambu County in central Kenya) (Figure 6). High genetic similarity coefficients were recorded for *Xam* isolates from Western and Coastal regions (Table 2) and hence high genetic homogeneity.

The genetic similarities observed among Kenyan strains isolated in different geographical locations (Western, Nyanza, Coast, Eastern and Central), demonstrate a clonal population structure for the pathogen. The lack of correlation between rep-PCR groupings and geographic origin of isolates is an indication that this bacterium has spread among different regions in Kenya. The results suggest that these isolates have not as yet diversified at the chromosomal level. The genetic diversity could not differentiate the yellow strain from the white and cream strains that cause cassava bacterial blight disease.

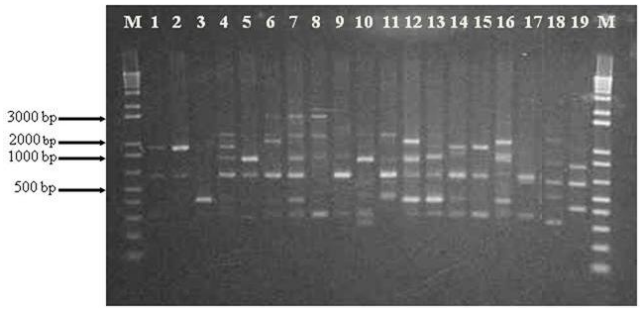

Fig. 6: Unweighted pair group method, arithmetic average (UPGMA) dendrogram constructed from rep-PCR fingerprinting data of *Xam* isolates from different regions in Kenya.

The random clustering of the *Xam* isolates from different geographical regions could be due to the continuity of the main cassava growing regions in Kenya. It is possible that a single isolate was introduced in Kenya and simultaneously spread to other parts.

Moreover, the farmer practice of obtaining plant materials from near and far, without taking precautions to avoid carrying infected stem cuttings and moving that to another place over a long distance could have perpetuated the same isolate. However, the exact source of the prevailing epidemic in Kenya still remains unknown. These results contradicts studies from other countries such as Columbia by Restrepo *et al*. [32] who reported that *Xam* causing bacterial blight exhibit a high level of genotypic variability and geographical differentiation.

Table 2: Similarity matrix of *Xam* isolates based on combined analysis of Jaccard matrix

	Xam1W	Xam24N	Xam19E	Xam26C	Xam8W	Xam6W	Xam2W	Xam22N	Xam27C	Xam30C	Xam25N	Xam10W	Xam16W	Xam15W	Xam5W	Xam21W	Xam23W	Xam4W	Xam11W	Xam25Ce
Xam1W	1	1	0	0.5	0.25	0.5	0.143	0	0.25	0	0.25	0.2	0.2	0.2	0.5	0.667	0.167	0.333	0.4	0.25
Xam24N		1	0	0.5	0.25	0.5	0.143	0	0.25	0	0.25	0.2	0.2	0.2	0.5	0.667	0.167	0.333	0.4	0.25
Xam19E			1	0	0	0	0.167	0	0	0	0.333	0.25	0.25	0.25	0.25	0	0.2	0	0.2	0.333
Xam26C				1	0.4	0.333	0.429	0.167	0.4	0.167	0.4	0.333	0.333	0.333	0.333	0.4	0.5	0.2	0.5	0.167
Xam8W					1	0.4	0.5	0.2	1	0.5	0.2	0.75	0.75	0.75	0.4	0.5	0.6	0.667	0.333	0.5
Xam6W						1	0.429	0.4	0.4	0.167	0.167	0.333	0.333	0.333	0.6	0.75	0.286	0.5	0.286	0.4
Xam2W							1	0.5	0.5	0.286	0.5	0.667	0.667	0.667	0.429	0.286	0.833	0.333	0.375	0.5
Xam22N								1	0.2	0.2	0.2	0.167	0.167	0.167	0.167	0.2	0.333	0.25	0	0.2
Xam27C									1	0.5	0.2	0.75	0.75	0.75	0.4	0.5	0.6	0.667	0.333	0.5
Xam30C										1	0	0.4	0.4	0.4	0.167	0.2	0.333	0.25	0.333	0.2
Xam25N											1	0.4	0.4	0.4	0.4	0.2	0.6	0.25	0.333	0.5
Xam10W												1	1	1	0.6	0.4	0.8	0.5	0.5	0.75
Xam16W													1	1	0.6	0.4	0.8	0.5	0.5	0.75
Xam15W														1	0.6	0.4	0.8	0.5	0.5	0.75
Xam5W															1	0.75	0.5	0.5	0.5	0.75
Xam21W																1	0.333	0.667	0.333	0.5
Xam23W																	1	0.4	0.429	0.6
Xam4W																		1	0.167	0.667
Xam11W																			1	0.333
Xam25Ce																				1

4. CONCLUSION

In conclusion, the results of this study demonstrate that *Xam* is widespread in all cassava growing regions of Kenya. The presence of this pathogen in the different cassava growing regions of Kenya should be considered as significant threat to food security particularly among the subsistence and smallholder cassava farmers. Analyses done using rep-PCR reported here provides the first data on the molecular characteristics of *Xam* infecting cassava in Kenya, and perhaps not yet reported in other parts of the world. The results also demonstrated a very high genetic relatedness within *Xam* isolates that were isolated on cassava from Kenya. From this, one can speculate that the current *Xam* populations in Kenya have evolved from the same origin, and this, may prove useful in future breeding programmes. 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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