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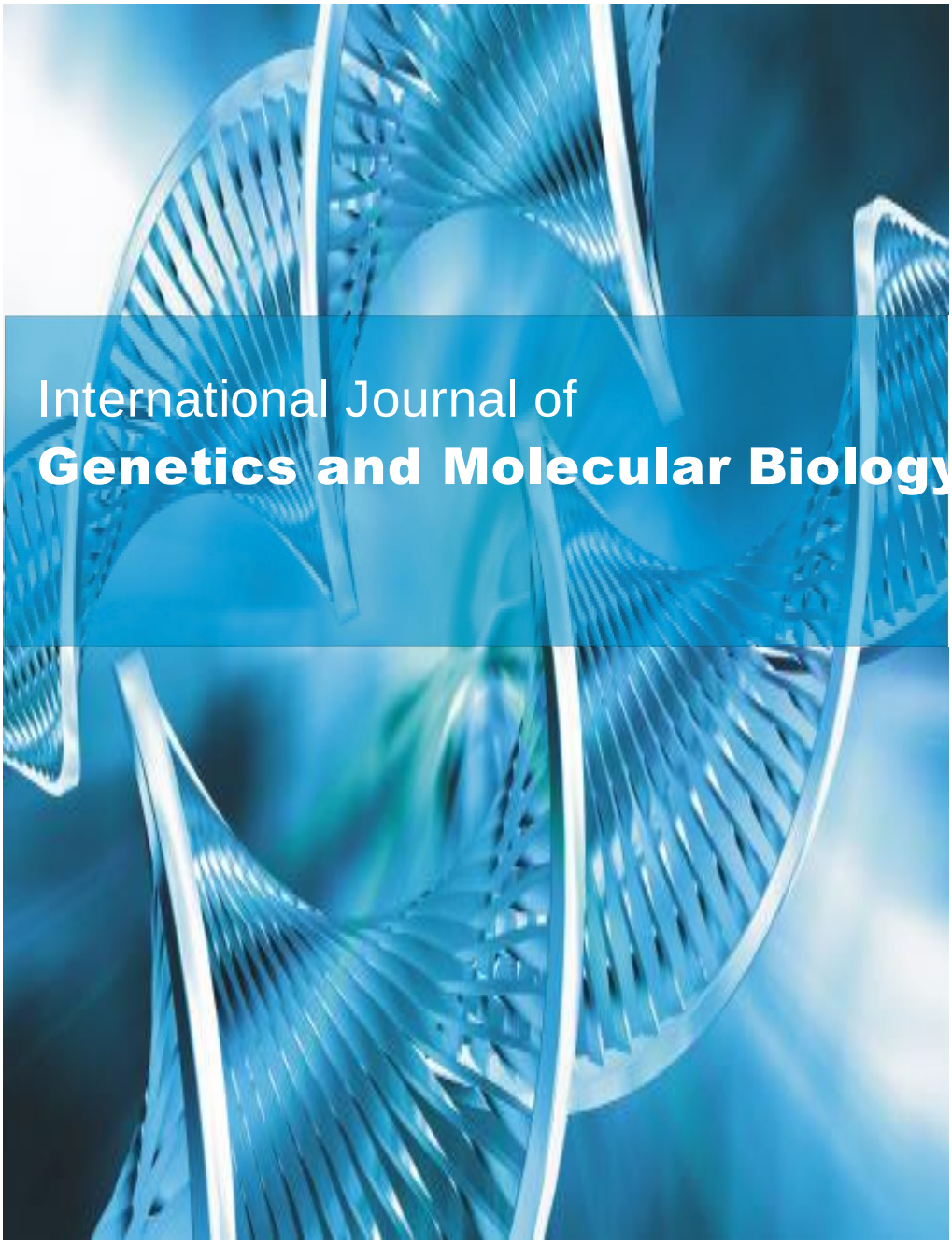

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Full Length Research Paper

Genetic analysis of twenty two selected genotypes of *Jatropha curcas* L. (physic nut) from Africa, Asia and America, using SSR and AFLP markers

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Twenty two genotypes of *J. curcas* L. from Africa (Senegal, Burkina Faso, Mali, Congo and Madagascar Island), Asia (Cambodia, China and India) and America (Ecuador, Dominican Republic and Brazil) selected for their vigor and their productivity were analyzed with ten SSR primer pairs and six AFLP primer combinations. The two marker approaches showed their ability to effectively reveal polymorphism among the selected genotypes: 94.02 and 56% of polymorphism for AFLPs and SSRs respectively. Among the three groups of selected genotypes, the Asian group was the least diverse while the genetic diversities found in African and American groups were slightly comparable. The Nei's genetic diversity (He) of all twenty-two selected genotypes was 0.2029 based on combined SSR+AFLP data. The Gst value and the AMOVA analysis indicated that more than 80% of the genetic diversity resided within the groups. The analysis of the genetic relationships between the genotypes using the Nei's standard dissimilarity matrix gave dissimilarity coefficients ranging from 0.14397 to 0.73943 with an average of 0.3540. The most distant genotypes were found between a genotype from Africa (Congo) and one from America (Ecuador). The clustering of genotypes obtained with the neighbor-joining dendrogram and the PCoA analysis revealed the existence of a certain level of diversity that can be used by breeders.

Key words: Biodiesel, genetic diversity, jatropha, molecular markers, AFLP, SSR, plant breeding.

INTRODUCTION

Jatropha curcas L. is a small perennial tree native to tropical America that has spread to tropical and subtropical regions of Asia and Africa over the last 200 years (Marinho et al., 2018). International attention to this plant has emerged during the past decade from the need to reduce dependence on the increasing scarcity and

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cost of fossil fuels and their associated carbon emissions (Moniruzzaman et al., 2017). Jatropha has been identified as a promising candidate for clean and renewable energy because of its seed oil which had the characteristics of diesel, its non-food nature and its supposed capability of high yield on marginal soils with minimum management requirements and inputs (Vásquez-Mayorga et al., 2017; Cardoso et al., 2018). For these reasons, jatropha was assumed to have potential to bring socio-economic benefits and there has therefore been a heightened global interest in large-scale jatropha cultivation and many jatropha projects have been implemented in Asia, Africa and Latin America (Soto et al., 2018). However, the performance of this new crop has been very disappointing despite claims of high yields, drought tolerance, low nutrient requirements, high returns on investment etc.; jatropha investments failed to meet global expectations (Antwi–Bediako et al., 2019). Consequently, doubts have been cast on the profitability and the financial viability of its cultivation, leading to the disillusionment of farmers and disadoption of jatropha by most of them (Soto et al., 2018; Vandepitte et al., 2019).

One of the main causes of the problem of jatropha cultivation was the low seed yield (Moniruzzaman et al., 2017; Soto et al., 2018). The cultivars used did not achieve the initially anticipated high seed yields, because the expectations were not based on field performance and validated scientific knowledge (Govender et al., 2018). The promotion was based on a multitude of unfounded claims, which did not lead to a commercially successful jatropha production.

Actually, *J. curcas* is an incipiently domesticated species with no availability of competitive commercial cultivars (Díaz et al., 2017). It is mostly a xenogamic plant, highly heterozygous for most characteristics, which implies a high degree of segregation (Díaz et al., 2017). The less productivity observed was because of unavailability of suitable high yielding varieties and a good commercial variety is still missing (Cardoso et al., 2018). As for all non-domesticated perennials, time is needed for the development of commercial varieties of jatropha; unfortunately this fact seems to have been overlooked and jatropha has been considered a commercial crop without any real scientific basis.

According to Tjeuw (2017), Jatropha has the potential to be a cash-crop like cotton or rubber (*Hevea brasiliensis* M.) which are both equally non edible but seem to be well accepted and not seen as a threat to food production. However for the successful exploitation of jatropha, there is an urgent need for improved genetic material with known yield characteristics over a wide range of climatic and agro-ecological conditions. It is one of the main effective solutions and the future challenge for jatropha breeders (Díaz et al., 2017; Moniruzzaman et al., 2017; Cardoso et al., 2018). This will require extensive plant breeding and molecular strategies to investigate systematically and scientifically jatropha potential for the development of a competitive commercial variety (Tjeuw, 2017).

In recent years, works on the genetic improvement of the species have been undertaken but has not yet resulted in improved competitive cultivars (Montes and Melchinger, 2016; Peixoto et al., 2017; Cardoso et al., 2018). For example, in 2018, Cardoso et al. from the *J. curcas* plant breeding program of the Federal University of Viçosa (Brazil) reported the selection of 20 promising individuals for crosses and for cloning aiming to bring genetic gains for successful cultivation. Govender et al. (2018) for their part identified genes with a putative implication in biological processes linked to yield, which could be of great importance in breeding strategies and subsequent production of superior and efficient *J. curcas* varieties.

The present study deals with twenty-two genotypes selected for their production and vigor from a collection of *J. curcas* from Africa, Asia and America, and planted on an experimental farm located in Bokhol (Senegal). For the development of an efficient breeding program, the determination of genetic relationships among these selected genotypes is critical (Díaz et al., 2017). It is an initial step towards efficient parental selection and breeding of superior genotypes. The present study was undertaken to determine and understand the genetic relationships between the selected genotypes, using SSR and AFLP markers in order to propose the best crossing scheme for a breeding program.

MATERIALS AND METHODS

Plant materials

The plant material was provided by the Laboratory of Tropical Agroecology of Gembloux Agro-Bio Tech (University of Liège, Belgium). It included dried leaves of twenty-two genotypes of *J. curcas* (Table 1) originating from Africa (Senegal, Burkina Faso, Mali, Congo and Madagascar), Asia (Cambodia, China and India) and America (Ecuador, Dominican Republic and Brazil). These genotypes were selected according to several parameters but especially on the basis of their productivity and their good vigor from a collection of *J. curcas* grown on an experimental farm located in Bokhol in Senegal.

DNA isolation

Total genomic DNA was isolated from 20 mg of dried leaves following a slightly modified mixed alkyltrimethylammonium bromide (MATAB) method as described by Lacape et al. (2003). Briefly, leaves were ground in 2 ml safe-lock microtubes (Eppendorf, Belgium) with stainless steel beads using a TissueLyser mixer-mill (Qiagen, Belgium) and dissolved in 800 µL of MATAB buffer (0.1 M Tris HCl, pH 8.0, 1.5 M NaCl, 20 mM EDTA, 2% MATAB, 1% polyethylene glycol 6000, 0.5% sodium sulphite) at 72°C. The

Table 1. Identification number, country and region of origin of the studied genotypes of *J. curcas*.

N°	Collection identity	Country	Region
1	TA4(13/25)	Senegal	Africa
2	YE4	Senegal	Africa
3	BUR2	Burkina Faso	Africa
4	BA5(1/21)	Burkina Faso	Africa
5	MA3(13/43)	Mali	Africa
6	CMA1	Congo	Africa
7	CMI6	Congo	Africa
8	CMS2	Congo	Africa
9	CMU3	Congo	Africa
10	CON3	Congo	Africa
11	MAD4	Madagascar	Africa
12	MA4	Madagascar	Africa
13	CAM4	Cambodia	Asia
14	CH2	China	Asia
15	IN3	India	Asia
16	INH1	India	Asia
17	INP1	India	Asia
18	MG2	Brazil	America
19	NN2	Brazil	America
20	VE2	Brazil	America
21	EQ2	Ecuador	America
22	RD3	Dominican Republic	America

samples were incubated for 1 h at 72°C and cooled for 5 min at room temperature. Then, 960 µL of chloroform-isoamyl alcohol (CIA) (24:1) was added to each sample. All samples were homogenized by inversion for 5 min, before centrifugation at 6200 g for 20 min at room temperature. The supernatant (800 µL) was collected and the DNA was precipitated with 640 µL of isopropanol by gentle shaking. After a centrifugation at 6200 g for 20 min, the pellet was washed with 600 µL of 70% ethanol through a centrifugation at 6200 g for 10 min, dried at 60°C for 10 min and dissolved in 100 µL of TE buffer (10 mM Tris HCl, pH 8.0,1 mM EDTA). RNA was removed by RNAse treatment at 37°C for 1 h. DNA quality and concentration were evaluated by electrophoresis in SYBR Safe (Invitrogen) stained with 1% agarose gels through comparison with low DNA mass ladder (Invitrogen, Belgium). Final concentration of DNA was adjusted to 50 ng μL^{-1} for use in PCR analysis.

SSR analysis

Ten SSR primer pairs (jcds 10, jcds 24, jcds 58, jcds 66, jcps 1, jcps 6, jcps 20, jcps 21, jcms 2 and jcms 30) were selected from the study of Pamidimarri et al. (2009). Polymerase chain reactions (PCR) were performed in 10 µL volume containing approximately 25 ng of template DNA, 0.6 U of Taq DNA Polymerase, 2.5 mM $MgCl_2$, 1x Polymerase Buffer, 2 µM of each forward and reverse primers, and 0.2 mM of dNTPs mix. A PTC-200 thermal cycler (BioRad, Belgium) was used, with a PCR conditions consisting of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final 72°C extension for 8 min. Amplification products were separated on 6.0% denaturing polyacrylamide gel and visualized by silver stain according to the following protocol: Fixing the gel for 3 min in a fixing solution (10% ethanol, 0.5% acetic acid, in water), staining for 5 min in a staining solution (0.2% silver nitrate, in water), rinsing in de-ionized water for 3 s, developing in a cold (4–10°C) developer solution (0.15% sodium hydroxide, 0.2% formaldehyde, in water) until the DNA bands became visible. The gel was rinsed for 1 min in the fixing solution, air-dried and the microsatellite bands were photo-documented and analyzed.

AFLP analysis

Six AFLP primer combinations were used (E-AAG/M-CAC, E-ACT/M-CAT, E-AAC/M-CTT, E-ACA/M-CAT, E-ACG/M-CTC and E-AGC/M-CAA). AFLP was carried out using the "AFLP Analysis System I / AFLP starter primer kit" (Invitrogen, Belgium) following the protocol proposed by Invitrogen. Briefly, genomic DNA (250 ng) was double digested with *Eco*R I and *Mse* I restriction endonucleases. The digested DNA fragments were ligated to *Eco*R I and *Mse* I adaptors with T4 DNA ligase to generate template DNA for amplification by PCR. Two consecutive PCR were performed: A pre-selective and selective PCR. In the pre-selective reaction, DNA was amplified using an AFLP pre-amp primer pair complementary to the adaptors and each having one selective nucleotide. Pre-selective PCR amplification was used as template for the selective

Table 2. Details of the amplified bands obtained from the DNA samples of the twenty two studied genotypes of *J. curcas*, using SSR and AFLP markers.

Marker type	Primer	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	% of polymorphic bands
SSR	jcds 10	8	3	5	37.5
	jcds 24	5	4	1	80
	jcds 58	7	4	3	57.14
	jcds 66	10	4	6	40
	jcps 1	3	0	3	0
	jcps 6	3	1	2	33.33
	jcps 20	9	8	1	88.89
	jcps 21	13	6	7	46.15
	jcms 2	6	4	2	66.67
	jcms 30	11	8	3	72.73
	Total	*75*	*42*	*33*	*56*
	Mean	***7.5***	***4.2***	***3.3***	***56***
AFLP	EAAG/MCAC	25	21	4	84
	EACT/MCAT	52	51	1	98.08
	EAAC/MCTT	81	76	5	93.83
	EACA/MCAT	64	62	2	96.87
	EACG/MCTC	53	47	6	88.68
	EAGC/MCAA	93	89	4	95.70
	Total	*368*	*346*	*22*	*94.02*
	Mean	***61.33***	***57.67***	***3.67***	***94.02***

amplification using AFLP primers, each containing three selective nucleotides. The amplified Fragments were resolved using 6.0% denaturing polyacrylamide gels and DNA bands were visualized by silver staining, as described previously.

Data collection and statistical analysis

The scoring of bands was done as present (1) or absent (0) for each SSR and AFLP marker allele and data was entered in a binary data matrix as discrete variables. POPGENE software (version 1.32) was used to calculate observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (He) and Shannon's information index (I). Within species diversity (Hs), total genetic diversity (Ht), mean coefficient of gene differentiation (Gst) and estimate of gene flow (Nm) were also calculated by POPGENE software. Dissimilarity coefficients between the genotypes, in a pairwise comparison, were computed using the Nei's standard dissimilarity coefficient (Ds) to estimate relationships between the genotypes studied. The resulting dissimilarity matrix was subjected to cluster analysis by Neighbor-joining method and a dendrogram, showing the distance-based interrelationship among the genotypes, was generated using FreeTree software (Pavlicek et al., 1999) and the drawing program TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Analysis of molecular variance (AMOVA) was carried out with GenALEx 6.5 software (Peakall and Smouse, 2001) to examine total genetic variation among and within accessions. In addition, Principal Component Analysis (PCoA) was performed with the same software, in order to more effectively view the patterns of genetic distance. GenALEx software was also used to calculate Nei's unbiased genetic distance among the different populations. Nei's unbiased genetic distance is an accurate estimate of the number of gene differences per locus when populations are small (Nei, 1978). Finally, a Mantel test was achieved with the GenALEx software to reveal the correlation between the two techniques (SSR and AFLP).

RESULTS

Polymorphism of SSR and AFLP amplified products

All the SSR primers and the AFLP primer combinations used amplified fragments across the twenty two genotypes studied (Table 2). In total, 75 bands were revealed for the 10 SSR primers. The number of bands per primer ranged from three (jcps 1 and jcps 6) to thirteen (jcps 21), with an average of 7.5 bands per primer. All the primers showed different levels of polymorphism except jcps 1 which showed no polymorphism among the twenty two jatropha genotypes. The percentage of polymorphic bands per primer ranged from 0% (jcps 1) to 88.89% (jcps 20), with an average of 56%. Four primers out of the 10 primers showed less

Table 3. Genetic variability across the three groups of *J. curcas* investigated using SSR primers.

Pop	Observed no. of alleles (Na)	Effective no. of alleles (Ne)	Nei's gene diversity (He)	Shannon's information index (I))
Africa	1.3467 (0.4791)	1.1928 (0.3210)	0.1147 (0.1788)	0.1733 (0.2602)
Asia	1.1200 (0.3271)	1.1031 (0.2919)	0.0541 (0.1510)	0.0766 (0.2123)
America	1.4933 (0.5033)	1.3793 (0.4359)	0.2053 (0.2249)	0.2959 (0.3163)
Africa+Asia+America	1.5600 (0.4997)	1.2347 (0.3060)	0.1490 (0.1709)	0.2354 (0.2503)

The values in the brackets are standard deviation.

Table 4. Genetic variability across the three groups of *J. curcas* investigated using AFLP primers.

Pop	Observed no. of alleles (Na)	Effective no. of alleles (Ne)	Nei's gene diversity (He)	Shannon's Information index (I))
Africa	1.7962 (0.4034)	1.3503 (0.3571)	0.2122 (0.1816)	0.3318 (0.2475)
Asia	1.4565 (0.4988)	1.2637 (0.3585)	0.1552 (0.1915)	0.2345 (0.2760)
America	1.5924 (0.4921)	1.3162 (0.3530)	0.1910 (0.1872)	0.2924 (0.2688)
Africa+Asia+America	1.9402 (0.2374)	1.3519 (0.3600)	0.2139 (0.1769)	0.3405 (0.2310)

The values in the brackets are standard deviation.

than 50% polymorphic bands. For the six AFLP primer combinations, a total of 368 bands were produced. The number of bands per primer combination ranged from 25 (*EAAG/MCAC*) to 93 (*EAGC/MCAA*) with an average of 61.33 bands per primer combination (Table 2). Of the 368 AFLP bands produced, 346 bands were polymorphic, accounting for 94.02%. The percentage of polymorphism per primer combination ranged from 84% (*E-AAG/M-CAC*) to 98.07% (*E-ACT/M-CAT*).

Genetic variability revealed with single-population descriptive statistics

At the level of single-population descriptive statistics, SSR results (Tables 3) revealed that the group of American genotypes had the highest values for observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (He), Shannon's information index (I) with 1.4933, 1.3793, 0.2053 and 0.2959 respectively. The group of Asian genotypes showed the lowest values for these parameters (Na = 1.12, Ne = 1.1031, He = 0.0541 and I = 0.0766), while the group of African genotypes presented intermediate values (Na = 1.3467, Ne = 1.1928, He = 0.1147 and I = 0.1733). The values of these parameters for all the twenty two genotypes were Na = 1.5600, Ne = 1.2347, He = 0.1490 and I = 0.2354.

With AFLP data (Table 4), the highest values for Na, Ne, He and I were found in African genotypes (Na = 1.7962, Ne = 1.3503, He = 0.2122 and I = 0.3318), followed by American (Na = 1.5924, Ne = 1.3162, He = 0.1910 and I = 0.2924), and then Asian (Na = 1.4565, Ne = 1.2637, He = 0.1552 and I = 0.2345). For all the 22 genotypes the results were Na = 1.9402, Ne = 1.3519, He = 0.2139 and I = 0.3405.

With the combined SSR+AFLP data (Table 5), the highest values for Na, Ne, He and I were found in African genotypes (Na = 1.7201, Ne = 1.3236, He = 0.1957 and I = 0.3050), followed by American (Na = 1.5756, Ne = 1.3269, He = 0.1934 and I = 0.2930), and then Asian (Na = 1.4565, Ne = 1.2637, He = 0.1552 and I = 0.2345). The values of these parameters for the twenty two genotypes were Na = 1.8758, Ne = 1.3321, He = 0.2029 and I = 0.3227.

Genetic diversity revealed with multi-populations descriptive statistics

Results of multi-populations descriptive statistics are presented in Table 6. The values for total genetic diversity among the groups (Ht) was 0.1586 for SSR, 0.2095 for AFLP and 0.2009 for SSR+AFLP, while within groups diversity (Hs) was found to be 0.1247, 0.1861 and 0.1757 for SSR, AFLP and SSR+AFLP respectively. Mean coefficient of gene differentiation (Gst) was 0.2152 with SSR data, indicating that 21.52% of the total genetic variation in the groups occurred among groups while 78.48% of the genetic diversity resided within groups. With AFLP and SSR+AFLP data, Gst value was 0.1113 and 0.1252 respectively, indicating that 88.87% (for

Table 5. Genetic variability across the three groups of *J. curcas* based on combined SSR+AFLP primers.

Pop	Observed no. of alleles (Na)	Effective no. of alleles (Ne)	Nei's gene diversity (He)	Shannon's information index (I))
Africa	1.7201 (0.4495)	1.3236 (0.3558)	0.1957(0.1846)	0.3050 (0.2564)
Asia	1.3995 (0.4904)	1.2365 (0.3530)	0.1381 (0.1890)	0.2078 (0.2726)
America	1.5756 (0.4948)	1.3269 (0.3685)	0.1934 (0.1939)	0.2930 (0.2770)
Africa+Asia+America	1.8758 (0.3301)	1.3321 (0.3539)	0.2029 (0.1774)	0.3227 (0.2374)

Table 6. Values of parameters of multi-populations genetic variability obtained in the study of the three groups of *J. curcas*, based on SSR and AFLP markers.

Marker type	Total gene diversity (Ht)	Intra-population gene diversity (Hs)	Coefficient of gene differentiation (Gst)	Estimate of gene flow (Nm)
SSR	0.1586 (0.0320)	0.1247 (0.0224)	0.2152	1.8234
AFLP	0.2095 (0.0310)	0.1861 (0.0245)	0.1113	3.9920
SSR + AFLP	0.2009 (0.0315)	0.1757 (0.0246)	0.1252	3.4931

The values in the brackets are standard deviation.

Table 7. Pairwise population matrix of Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) calculated with combined SSR+AFLP data obtained in the study of the three groups of *J. curcas*.

	Africa	Asia	America
Africa	0.0000	0.9793	0.9763
Asia	0.0209	0.0000	0.9618
America	0.0240	0.0390	0.0000

AFLP) and 87.48% (for SSR+AFLP) of the genetic diversity resided within groups.

Analysis of molecular variance (AMOVA) partitioned the total genetic variance into variance among groups and within groups. The results of AMOVA based on SSR, AFLP and SSR+AFLP data showed respectively 5, 0 and 1% of molecular variance among groups, while within the groups these values were 95, 100 and 99%.

The Nei's unbiased measures of genetic similarity (GS) and genetic distance (GD) among the three studied groups of *J. curcas* were calculated using SSR+AFLP data (Table 7). The GD of the three groups of genotypes varied from 0.0209 to 0.0390, with an average of 0.0279. The largest GD (0.0390) was found between the American and Asian groups and the smallest (0.0209) between African and Asian groups.

Genetic relationships among the genotypes and cluster analysis

Genetic relationships among the genotypes of the three groups of *J. curcas* were constructed by neighbor-joining cluster analysis based on the Nei's standard dissimilarity coefficients (Ds). GD matrixes were obtained from the SSR, AFLP and SSR+AFLP binary data. The Nei's standard dissimilarity coefficients (Ds) between the genotypes ranged from 0.00741 to 0.36682 with a mean of 0.08404 for SSR. The lowest dissimilarity coefficient was between the genotype#10 from Africa (Congo) and the genotype#19 from America (Brazil), and the highest was between genotype#21 from America (Ecuador) and genotype#22 from America (Dominican Republic). For AFLP, the Nei's standard dissimilarity coefficients ranged from 0.22044 to 1.22921 with a mean of 0.53718. The least dissimilar genotypes were genotype#12 from Africa (Madagascar) and genotype#17 from Asia (India) and the most dissimilar were genotype#9 from Africa (Congo) and genotype#11 from Africa (Madagascar). When the SSR and AFLP data were combined, the Nei's standard dissimilarity coefficients ranged from 0.14397 to 0.73943 with a mean of 0.3540. The lowest coefficient was found between genotype#12 from Africa (Madagascar) and genotype#17 from Asia (India), and the highest between

Table 8. Genetic distance (GD) matrix obtained by Nei's standard distance (Ds) and neighbor-joining method, showing the relationships among the studied genotypes of *J. curcas* using combined SSR+AFLP data.

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22
#1																						
#2	0.229																					
#3	0.354	0.34																				
#4	0.176	0.257	0.334																			
#5	0.326	0.32	0.433	0.374																		
#6	0.327	0.353	0.379	0.371	0.317																	
#7	0.252	0.279	0.391	0.289	0.323	0.326																
#8	0.221	0.243	0.357	0.247	0.311	0.334	0.224															
#9	0.529	0.525	0.561	0.561	0.518	0.5	0.416	0.518														
#10	0.239	0.294	0.282	0.289	0.352	0.319	0.305	0.267	0.549													
#11	0.292	0.307	0.404	0.333	0.414	0.393	0.302	0.258	**0.616**	0.268												
#12	0.233	0.223	0.317	0.219	0.332	0.353	0.211	0.226	0.459	0.271	0.319											
#13	0.351	0.309	0.396	0.36	0.4	0.403	0.329	0.334	0.515	0.383	0.368	0.312										
#14	0.318	0.288	0.344	0.32	0.325	0.388	0.306	0.283	0.494	0.353	0.363	0.234	0.304									
#15	0.19	0.227	0.353	0.225	0.311	0.318	0.183	0.219	0.475	0.23	0.284	0.17	0.327	0.246								
#16	0.271	0.298	0.411	0.323	0.349	0.323	0.286	0.264	0.456	0.316	0.313	0.259	**0.385**	0.317	0.262							
#17	0.193	0.211	0.336	0.243	0.313	0.341	0.199	0.2	0.5	0.251	0.255	0.144	0.273	0.267	0.158	0.262						
#18	0.268	0.286	0.35	0.297	0.339	0.306	0.19	0.268	0.391	0.26	0.341	0.226	0.334	0.276	0.206	0.346	0.221					
#19	0.31	0.266	0.349	0.358	0.321	0.352	0.294	0.341	0.482	0.31	0.412	0.267	0.363	0.361	0.271	0.382	0.223	0.308				
#20	0.446	0.451	0.514	0.504	0.435	0.491	0.39	0.43	0.615	0.463	0.461	0.372	0.451	0.425	0.388	0.419	0.434	0.421	0.548			
#21	0.421	0.453	0.522	0.478	0.454	0.463	0.456	0.395	**0.739**	0.421	0.465	0.438	0.525	0.473	0.398	0.45	0.452	0.461	0.58	0.518		
#22	0.394	0.363	0.504	0.425	0.407	0.421	0.384	0.416	0.546	0.375	0.501	0.33	0.434	0.392	0.361	0.368	0.355	0.317	0.374	0.433	**0.604**	

genotype#9 from Africa (Congo) and genotype#21 from America (Ecuador). The GD matrixes generated by the combined SSR+AFLP data (Table 8) also showed that, the most genetically distant genotypes in the African group were genotype#9 from Congo and genotype#11 from Madagascar (Ds = 0.61553). In the American groups the most distant genotypes were genotype#21 from Ecuador and genotype#22 from Dominican Republic (Ds = 0.60396). And finally in the Asian group, the most distant genotypes were genotype#13 from Cambodia and genotype#16 from India (Ds = 0.38502).

The dendrograms (Figures 1 to 3) constructed, defining the genomic relationships among analyzed genotypes, grouped the 22 *J. curcas* genotypes into three main clusters (I, II and III). For SSR, the composition of each cluster (Figure 1) was: Eight genotypes for cluster I (2 from Asia, 1 from America and 5 from Africa), six genotypes for cluster II (3 from Asia, two from America and 1 from Africa) and eight genotypes for cluster III (2 from America and 6 from Africa). The clustering results for AFLP (Figure 2) was: Eight genotypes for cluster I (2 from Asia and 6 from Africa), four genotypes for cluster II (1 from Asia, 1 from America and 2 from Africa), ten genotypes for

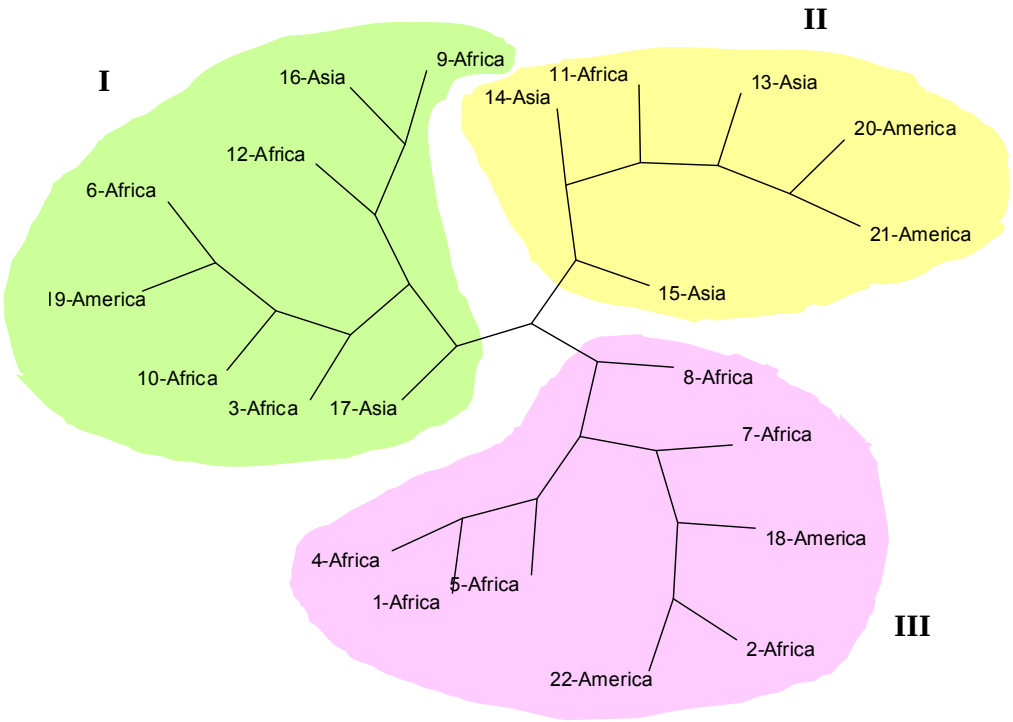

Figure 1. Dendrogram generated using Nei's standard distance (Ds) and neighbor-joining method, showing relationships between studied genotypes of *J. curcas*, based on SSR markers.

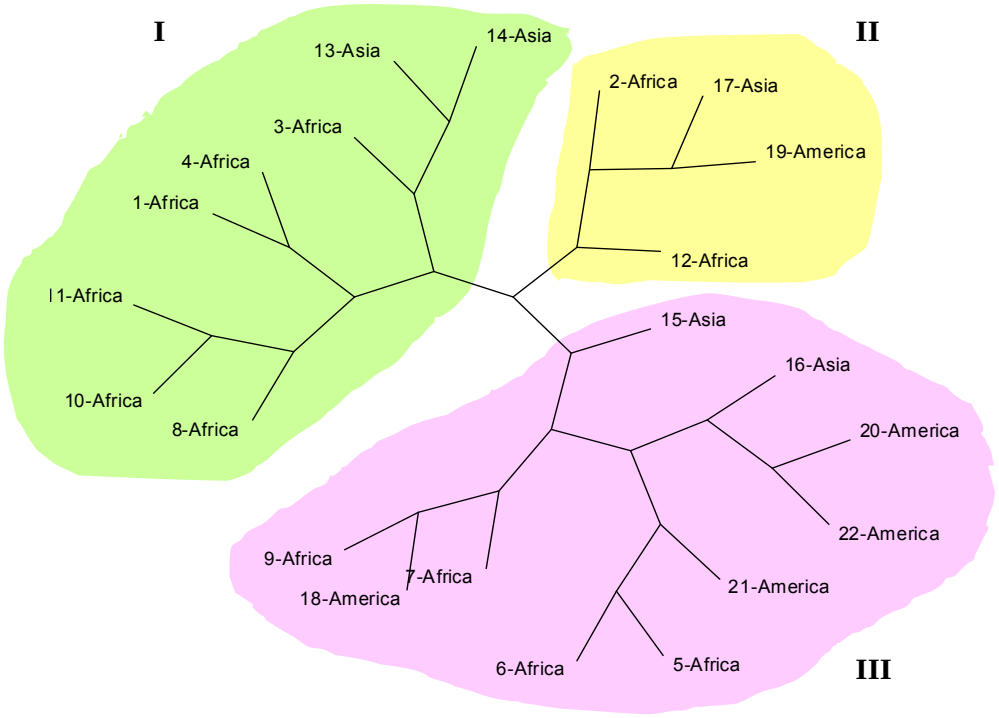

Figure 2. Dendrogram generated using Nei's standard distance (Ds) and neighbor-joining method, showing relationships between studied genotypes of *J. curcas*, based on AFLP markers.

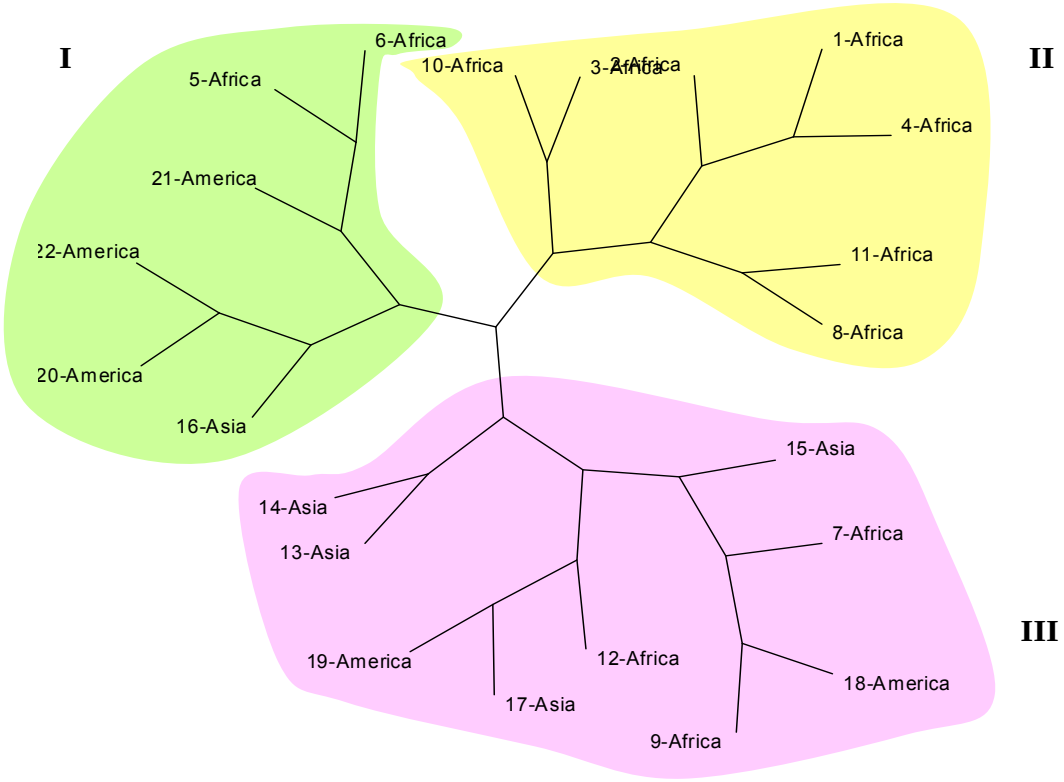

Figure 3. Dendrogram generated using Nei's standard distance (Ds) and neighbor-joining method, showing relationships between studied genotypes of *J. curcas*, based on AFLP+SSR markers.

cluster III (2 from Asia, 4 from America and 4 from Africa). For combined SSR and AFLP data, the clustering (Figure 3) was: Six genotypes for cluster I (2 from Africa, 1 from Asia and 3 from America), seven genotypes from Africa for cluster II and nine genotypes for cluster III (3 from Africa, 4 from Asia and 2 from America).

Principal Coordinate Analysis (PCoA) was used to add complementary information to the cluster analysis. The results of PCoA (Figure 4a, b and c) showed, clustering comparable to those of the dendrograms. No clear relation was shown between genotype associations and geographical origin.

A mantel test between the two Nei's genetic diversity indexes was performed with 99 permutations, in order to estimate correlations for the two molecular systems. It resulted in $r^2 = 0.1093$ (Figure 5).

DISCUSSION

In the present study, the two marker approaches (SSR and AFLP) showed their ability to effectively reveal polymorphism among plant materials of *J. curcas*. However, AFLP exceeded SSR in the ability to detect genetic polymorphism with higher resolving power (94.02% of polymorphic bands for AFLP against 56% for SSR). This might be due to the type of genetic polymorphism detected by each molecular marker system and the different type of information provided by each of them (Augustinos et al., 2016). Indeed, the higher multiplexing ability of arbitrarily dominant technologies such as AFLP is more efficient in detecting polymorphism per assay rather than high levels of polymorphism at each locus of the codominant SSR technology (Costa et al., 2016). Osorio et al. (2014) using a set of 29 SSR and 20 AFLP in a collection of *J. curcas*, found also higher percentage of polymorphism with AFLP (86%) than with SSR (73%).

The difference between the two marker systems was also shown by the Mantel test that revealed no significant associations between them ($r^2 = 0.1093$). A low correlation between AFLP and SSR has also been reported by Mardi et al. (2011) working on durum wheat, with $r^2 = 0.116$. According to Augustinos et al. (2016), poor correlation between genetic markers most likely indicates that they refer to different portion of the genome. Accordingly, the combined use of different marker systems may provide more reliable information

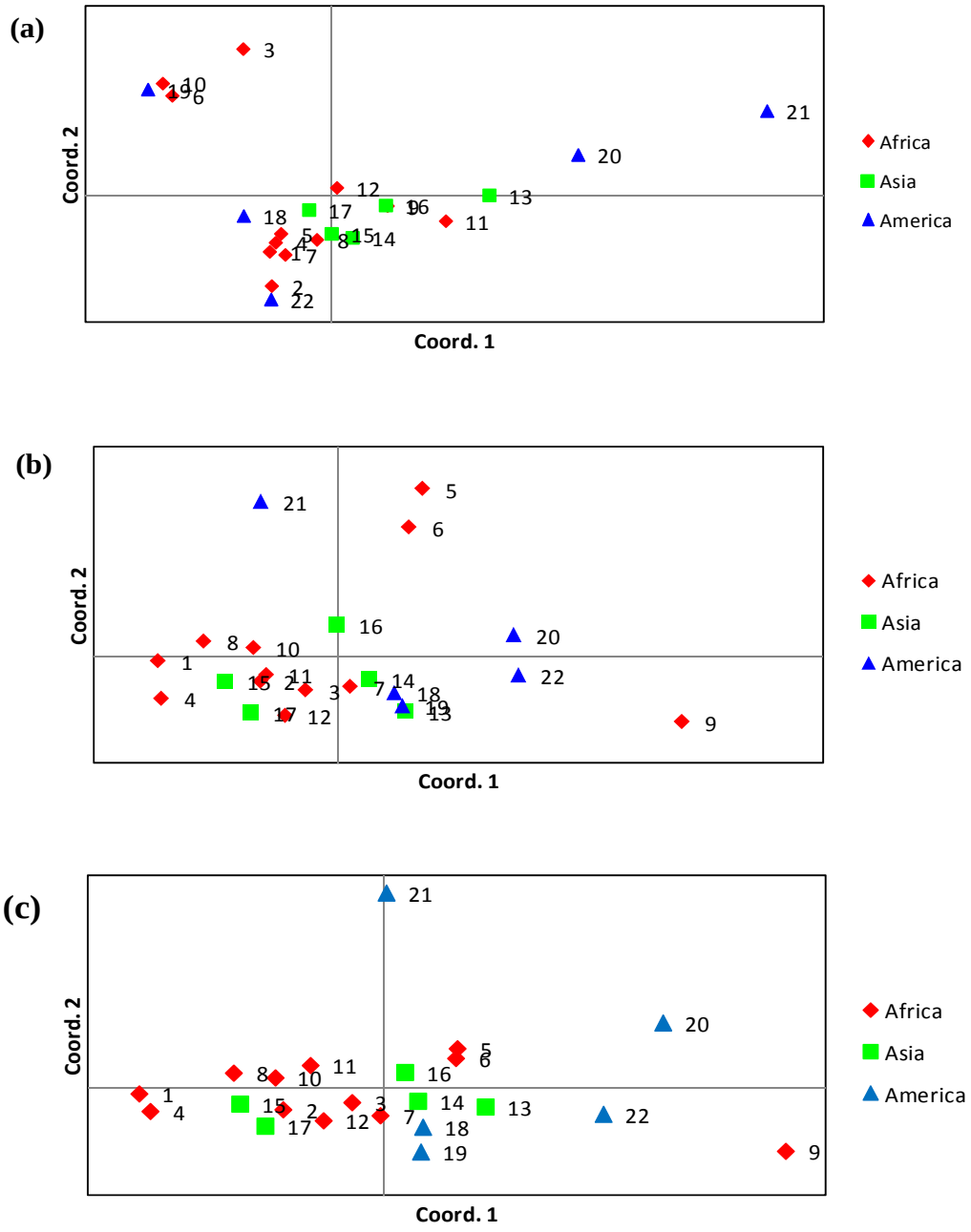

Figure 4. Two-dimensional plot of principal coordinate analysis (PCoA) of the twenty two studied genotypes of *J. curcas*, using SSR (a), AFLP (b) and combined SSR+AFLP (c) data. The numbers plotted represent genotype N°.

about genetic diversity when compared to the use of only one marker system (Leal et al., 2010; Augustinos et al., 2016). Therefore, the results obtained from the two marker systems used in the present study should be considered complementary.

With the combined data of the two markers systems, the values of Nei's genetic diversity obtained for the twenty two selected genotypes was He= 0.2029. This values was lower than those reported from populations of *J. curcas* in Chiapas in Mexico (He = 0.34-0.54) by Sanou et al. (2015). Considering that Chiapas (Mexico) is recognized as a center of origin and diversity of jatropha (Angel et al., 2016), the genetic diversity of the selected genotypes in the present study was not negligible.

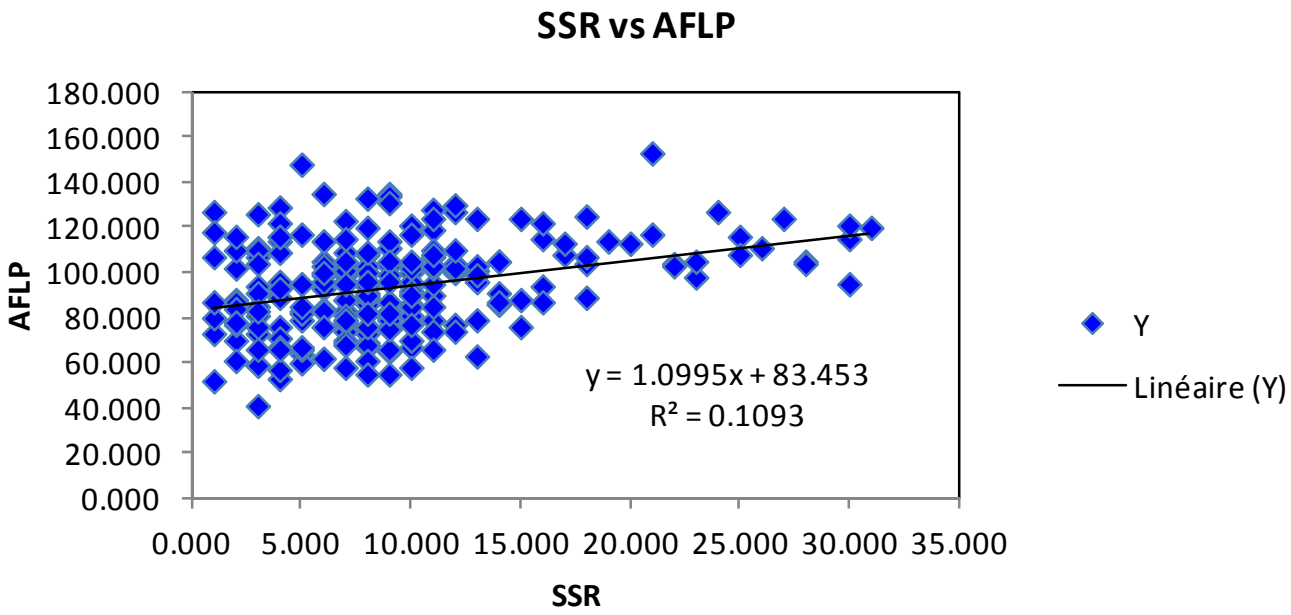

Figure 5. Output of mantel test comparing the AFLP and SSR genetic distance matrices.

Wright (1951) has previously shown that Gst is a valuable parameter to determine the degree of genetic differentiation between populations. For the selected genotypes, Gst value was 0.1252 with combined SSR+AFLP. This Gst value globally indicated that more than 87.48% of the genetic diversity resided within the groups and only 12.52% of the total genetic variation occurred among groups. These results agreed with the weak molecular variance among groups shown by AMOVA analysis and the low genetic distances found among the three groups of genotypes. Other studies in *J. curcas* based on different types of molecular markers have also reported that in general, there is limited variation among groups of *J. curcas* from various part of the world (Kumar et al., 2009; Subramanyam et al., 2009; Rosado et al., 2010). The low genetic differentiation among groups indicates that accessions of *J. curcas* have originated from the same genetic background, as mentioned by Pamidimarri and Reddy (2014) and Díaz et al. (2017).

Analysis of the genetic relationships between genotypes using the Nei standard dissimilarity matrix obtained from combined SSR + AFLP data, showed dissimilarity coefficients ranging from 0.14397 to 0.73943. This result indicated the existence of relatively important genetic distances between some genotypes. The most distant genotypes can be good candidates for crosses. The dendrogram, obtained from these genetic relationships among the genotypes, grouped the 22 *J. curcas* genotypes into three main clusters (I, II and III). Globally, the dendrogram showed three tendencies: (i) Genotypes from diverse regions group together in the same cluster, (ii) Genotypes from the same region scattered in different clusters, and (iii) Genotypes from the same region cluster together. This pattern of clustering showed no clear association between geographical distribution of genotypes and genetic diversity. This means that the geographical origin of the genotypes studied is not significantly related to their genetic similarity. Principal Coordinate Analysis (PCoA) confirmed this clustering pattern. Similar results were found by other authors such as Ambrosi et al. (2010), Maghuly et al. (2015) and Konan et al. (2018) showing that geographical origin do not necessarily reflect the genetic diversity of accessions of *J. curcas*. This lack of correspondence between the molecular classification and geographic origin of the accessions could be due to their possible common origin, confirming the hypothesis that *J. curcas* genetic stocks of Asia and African were introduced from America (Li et al., 2017; Gangapur et al., 2018).

The challenge of all plant genetic breeding programs is to maintain the genetic diversity within the target species while improving desired traits that enable plant materials to perform well (Díaz et al., 2017). The results obtained in this study indicate the existence of a certain level of diversity that can be used by breeders. The choice of the parents can be based on the distance existing between the different genotypes. Based on the Nei's standard dissimilarity matrix and the PCoA analysis using the

combined SSR+AFLP data, the greatest heterosis might be expected from the following crossing schemes. Cross on the one hand, the two most genetically distant genotypes from Africa (genotype#9 from Congo and genotype#11 from Madagascar); and on the other hand, cross the two most genetically distant genotypes from America (genotype#21 from Ecuador and genotype#22 from Dominican Republic). Then, select from each cross, the best F1 progenies by phenotypic performance. Finally, cross the selected African F1 genotypes to the selected American F1 genotypes and select their best progenies according to their phenotypic performances. These selected progenies can be multiplied by vegetative propagation to preserve excellent clonal genetic stocks in the heterozygous status or used for further selections by back crossing. Such crossings might allow for greater success in the production of genetic variability and thus might maximize the exploitation of heterosis and segregation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Impact of *glutathione S-transferase* genes polymorphisms on human papillomavirus infection and precancerous lesions in West African women

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Genetic polymorphisms of certain classes of glutathione S-transferase (GST), enzyme responsible for the biotransformation of drugs and xenobiotics, have been associated with risk of several cancers such as cervical cancer. The aim of this study is to investigate the impact of *glutathione S-transferase M1* and *T1* deletion on high-risk human papillomavirus (HR-HPV) infections and on dysplasia. A case-control study was carried out on 1069 endocervical samples from West African women including 482 HR-HPV positive and 139 patients had cervical lesions according to visual inspection with acetic acid and Lugol (VIA/VILI) screening. Deletion of the *GSTM1* and *GSTT1* genes was determined using conventional PCR and genotypes of HR-HPV by real-time PCR. An association with a reduced risk for HR-HPV infection was observed in Ivorian population with *GSTT1*-null (OR = 0.61, 95% CI = 0.40 - 0.92, p= 0.02) and *GSTM1*-active/*GSTT1*-null genotypes (OR = 0.56, 95% CI = 0.35 - 0.90, p= 0.02). In West African, women with *GSTT1*-null genotype had 1.72-fold higher risk for infection with HPV66 (p= 0.044) and reduced risk (OR = 0.39) for HPV35. Whereas women with *GSTM1*-null/*GSTT1*-active genotype had 2.32-fold higher risk for HPV18 infection (p= 0.042). *GSTT1*-null genotype was associated to cervical lesions in West African with a reduced risk (OR = 0.63, p= 0.017). The results of the present study demonstrate that *GSTT1*-null could be associated with cervical lesions and HPV35 infection with reduced risk. *GSTM1*-null associated with *GSTT1*-active could play a role in increasing the risk for HPV18 infection.

Key words: Cervical cancer, *GSTM1*, *GSTT1*, HR-HPV, West Africa.

INTRODUCTION

Cervical cancer is a major challenge for developing countries. The human papillomavirus (HPV) is considered as the main etiological agent responsible for cervical cancer (Walboomers et al., 1999). In 2018, the global incidence of cervical cancer was estimated at 570,000 cases with 311,000 deaths (Bray et al., 2018). About 85% of cases related to cervical cancer occur in low-income countries (Chuang et al., 2016; Randall and Ghebre, 2016). The disease was the fourth most common diagnosed cancer in women worldwide and the second in terms of incidence and mortality in developing countries (Bray et al., 2018). The highest incidence and mortality rates are recorded in Southern Africa followed by East and West Africa (Guinea, Burkina Faso and Mali) (Bray et al., 2018). According to WHO, cervical cancer related mortality might increase by 42% to reach 442,926 deaths in 2030 (WHO, 2015). The largest increase will occur in low- and middle-income countries and could be due to specific factors influencing HPV infection and cervical cancer in sub-Saharan Africa. Persistent HPV infections remain the main cause of precancerous lesions and cervical cancer (Walboomers et al., 1999; Schneider et al., 1992). Despite the fact that HPV infected women are at high risk for cervical cancer, not all infected women develop the disease as the infection is most often detected in asymptomatic people (Wheeler et al., 1993). Only a minority would develop cancer; the neoplastic lesions can regress spontaneously. Malignant transformation occurs during a period of 15 to 20 years in the infected epithelium of the cervix (Zur Hausen, 2002) and also involve the host genetic factors in the progression of the disease.

Among these genetic factors there is mounting evidence about glutathione S-transferase (*GST*) gene polymorphisms. Glutathione S-transferase (*GST*) is a polymorphic enzyme involved in the conjugation of reduced glutathione to harmful electrophilic compounds and in chemo-resistance to anticancer agents. Isoenzymes of *GST* are involved in detoxification of carcinogen and play a very important role in the cellular defense system. *GSTs* belong to the family of cytosolic enzymes (GST, EC 2.5.1.18) divided into 8 classes: *mu* (*GSTM*), *alpha* (*GSTA*), *pi* (*GSTP*), *theta* (*GSTT*), *zeta* (*GSTZ*), *sigma* (*GSTS*)), *kappa* (*GSTK*) and *omega* (*GSTO*) (Hayes and Pulford, 1995, Hayes and Mclellan, 1999). GST enzymes are soluble with a molecular mass of about 25 kDa. The most studied polymorphisms of *GST* genes consist of *mu*, *theta* and *zeta* classes and the subclasses are mainly *GSTM1*, *GSTT1*, *GSTP1*. The present study will focus on the polymorphisms of the *GSTM1* and *GSTT1* genes in West African women. So far, a single nucleotide polymorphism (G2619C at 534 position 7 exon) or a complete deletion of the gene was reported for *GSTM1* subclass with three known alleles: *GSTM1*A, GSTM1*B* and *GSTM1*0*. The first two alleles differ by a single nucleotide in exon 7 of the gene with no influence on the enzyme activity. The null allele *GSTM1*0* also called *GSTM1*-null results in the absence of the *GSTM1* enzyme. People with complete deletion of the *GSTM1* gene seem unable to metabolize epoxides or quinones (Hayes and Pulford, 1995; Hayes and Strange, 2000). The frequency of the null allele *GSTM1*0* is estimated at 50% in Caucasians and 27% in Asian population. *GSTT1* gene polymorphisms (A310C) result in the substitution of threonine residue into proline at position 104 of the amino acid sequence (Eaton and Bammler, 1999) with three alleles named *GSTT1*A*, *GSTT1*B* and *GSTT1*0*. The latter also called *GSTT1*-null differs from the first two alleles by an absence of enzymatic activity with a frequency estimated at 20 and 61% in Caucasian and Asian population respectively. Insufficient detoxification caused by gene polymorphism of the metabolizing enzymes or dysregulation in the elimination system of toxins (oxidative stress) from the body can lead to increased exposure to reactive carcinogenic derivatives and contribute to the malignant cellular reaction in women infected with HPV.

According to studies, oxidative stress was associated with an increase in viral replication in cells infected (Scholz et al., 1996), and the increase in oxidants has been associated with neoplastic progression of HPV16 (De Marco et al., 2012). Several studies in our laboratory have identified a number of high-risk HPVs associated with cervical lesions in West Africa other than 16 and 18 (Zohoncon et al., 2020). The importance of studying *GSTM1*, *GSTT1* genes deletion and high-risk HPV infection and precancerous lesions is very necessary. Several studies have investigated the association of *GSTM1* and *GSTT1* deletion in the acquisition of precancerous lesions and study cervical cancer in different countries with the exception of Africa. In this first pioneering study of a population of women from several West African countries, we have examined and verified the hypothesis that the deletions of *GSTM1* and *GSTT1* are associated with HR-HPV infection and the precancerous lesions.

MATERIALS AND METHODS

Ethical aspects

The women recruited gave their free and informed consent to participate in the study according to the Helsinki Declarations. The research protocol was approved by the Ethics Committee for Health

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Research (CERS) of Burkina Faso on January 10, 2018 with reference number 2018-01-012. The information obtained from the patient are kept strictly confidential. The results were used for a better therapy of women.

Type, site and study population

The study population consisted of 1,069 samples randomly selected from 2,133 endocervical samples collected from women in the general population of five West African countries, namely Benin, Burkina Faso, Côte d'Ivoire, Niger and Togo. HPV sampling and genotyping were carried out in 2017 as part of an earlier study funded by the *"Agence Universitaire de la francophonie"* (AUF). Ten cities were selected for sample collection in the five countries according to their importance in terms of population density and geographic location.

This is a cross-sectional, case-control study. We considered the high-risk HPV positive samples as the cases and the high-risk HPV negative samples as the controls. The distribution of the number of samples by city was: 99 cases and 111 controls chosen from 234 samples in Ouagadougou (Burkina Faso), 63 cases and 76 controls from 239 samples in Kara (Togo), 183 cases and 214 controls from 484 samples in Abidjan-Bouaké-Yamoussoukro (Côte d'Ivoire), 108 cases and 134 controls among 484 samples in Parakou-Cotonou-Borgou/Alibori-Abomey Calavi (Benin) and 29 cases and 52 controls chosen from 250 samples in Niamey (Niger). The sample size in each country was calculated according to the prevalence of HPV in the country. In total, there were 482 cases and 587 controls. Among the 1069 samples selected, 139 patients were carriers of lesions and 897 patients were without lesions according to the VIA/VILI tests.

Cervical specimen collection and screening for precancerous lesions

After sensitizing the study respondents about how to prevent HPV infection and cervical cancer risk, and obtaining free and informed consent from the women, a questionnaire was administered to the women to collect their socio-demographic, behavioural and clinical information. Endocervical swab samples were taken from the uterus of the respondents using a sterile cotton swab and a single use speculum; behind screening was done for visual inspection using acetic acid and Lugol (IVA/VILI) to determine cervical lesions or dysplasia in the women. An examination could specify the lesions types were not known. Patients with a positive VIA or VILI test were considered to have cervical lesions or dysplasia, and those with a negative VIA / VILI were considered to have normal cytology.
The samples obtained were immersed in a transport medium from the DNA-Sorb-A kit (Sacace Biotechnologies, Como, Italy) and stored at -20°C in the laboratory of the various sites. In CERBA/LABIOGENE (Pietro Annigoni Biomolecular Research Center / Molecular Biology and Genetics laboratory) of Ouagadougou (Burkina Faso), DNA was extracted, high-risk HPV genotypes were characterized and *GSTM1* and *GSTT1* deletion was done.

DNA extraction

The DNA of the endocervical samples was extracted using the commercial kit called "DNA-Sorb-A" from sacace biotechnologies® according to the manufacturer's protocol. The extracted DNA was stored at a temperature of -20°C in order to carry out PCR amplifications.

HR-HPV detection

HR-HPV was detected with the HPV Genotypes 14 Real-TMQuant kit (SACACE Biotechnologies®, Italy) using real-time multiplex PCR test for detection of 14 high-risk genotypes (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). For PCR amplification, each DNA sample, to be amplified was distributed in 4 tubes at the rate of 10 µl/tube. Each tube previously contained 15 µl of the reaction mixture and target specific genotypes. Positive and Negative controls given by the supplier have been performed following the same procedure.

The reaction mixture of 15 µl in the 4 tubes was composed of a mixture of Hot Start DNA, PCR-buffer-FRT and respectively the primers L1, E6 and E7 of the target regions of 3 to 4 HR-HPV and internal control (PCR-mix-1 16, 18, 31, IC; PCR-mix-1 39, 45, 59, IC; PCR-mix-1 33, 35, 56, 68; PCR-mix-1 51, 52, 58, and 66). The amplification program was 1 cycle of 95°C for 15 min, followed by 5 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 15 s and finally 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 15 s. The results were interpreted using Microsoft Excel program software called HPV Genotypes 14 Real-TM.xls (SACACE Biotechnologies®, Italy) according to the manufacturer's protocol.

GSTM1 and GSTT1 polymorphisms characterization

The method used for the genotyping of *GSTM1* and *GSTT1* is a conventional multiplex PCR described by Chen et al. (Chen et al., 1997). The primers used were _F': 5'GAACTCCCTGAAAAGCTAAAGC-3' and R' : 5'GTTGGGCTCAAATATACGGTGG-3' for GSTM1; _F' : 5'-TTCCTTACTGGTCCTCACATCTC-3' and R' : 5'-TCACCGGATCATGGCCAGCA-3' for GSTT1 ; _F ': 5'CAACTTCATCCACGTTCACC-3' and R' : 5'GAAGAGCCAAGGACAGGTAC-3' for internal control (β-globine). Each well contained 10µl of Taq Gold 360 Master Mix Ampli, 1µl of each primer, 7µl of sterile water, 2µl of DNA. The PCR amplification program began with a denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for one min, 57°C for 1 min and 72°C for one minute, ending with an extension at 72°C for 7 min.

PCR amplification products were revealed by 3% agarose gel electrophoresis containing ethidium bromide. The bands of 215 bp, 480 bp and 268 bp were allocated to *GSTM1*, *GSTT1*, and β-globin, respectively (Figure 1). The absence of PCR products corresponding to *GSTM1*, *GSTT1* was considered to be zero genotype and invalid PCR in the absence of PCR products corresponding to β-globin.

Statistical analysis

Our data were analyzed by Excel 2016 software, SPSS Statistics 25.0.0.0, Epi Info 7.2.2.6. The confidence interval was set at 95% and the fisher test was used for the comparison. The difference was statistically significant for $p < 0.05$.

RESULTS

Sociodemographic characteristic of the study population

The present study concerned 1069 samples. Table 1 shows the sociodemographic characteristic of the study population. Their ages range from 15 to 72 years with a mean of 35.48 ± 9.6 years. The median age was 35 years. 25-34 and 35-44 age groups were the most

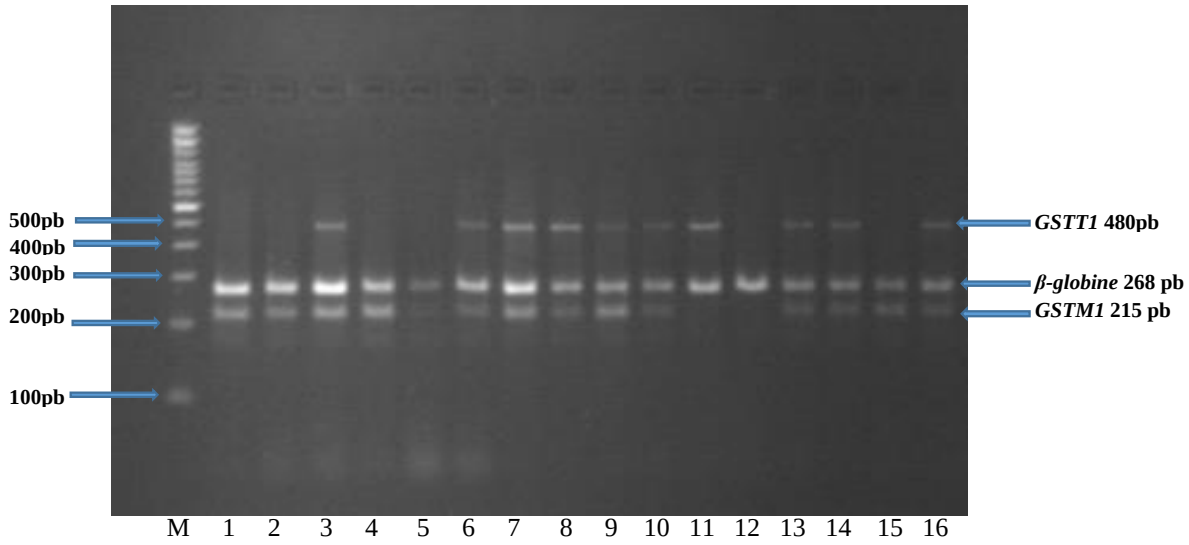

Figure 1. PCR-Multiplex Electrophoresis Gel. M: Molecular weight marker (100 bp). Samples 5 and 12: Double genotypes null of *GSTM1* and *GSTT1*. Samples 1; 2; 4 and 15: Genotypes of *GSTM1* present and *GSTT1* null. Samples 3; 6; 7; 8; 9 and 10: Double present genotypes of *GSTM1* and *GSTT1*. Sample 11: *GSTM1* null and GSTT1 present.

represented with frequencies of 36.30 and 35.45%, respectively. The study population was made of 64.36% of women who were less than 30 years old. The married wives in our study represented 71.66% of the study population. Women who had their first sexual intercourse between 15 and 24 years old represented 78.77% (842/1069). The number of sexual partners ranged from zero to more than two. About 29% of women said they were on contraception, 13.94% had contracted a sexually transmitted infection during their life, 0.84% said they were diagnosed with HIV. VIA/VILI tests were negative in 86.16% of the study population. HR-HPV infections accounted for 45.09% (28.16 single infections and 16.93% multiple infections). The most frequent HR-HPV in cases and controls were HPV 52 (Table 2)

GSTM1, GSTT1 polymorphism and HPV infection in western Africa

In our study 29 (2.71%) samples were considered invalid after PCR because no band was detected during electrophoresis migration. One thousand and forty (1040) samples produced valid results during PCR testing. Table 3 shows *GSTM1* and *GSTT1* and HR-HPV infection in West Africa. The presence of the *GSTM1* and *GSTT1* was observed in 74.52 and 55.87% of the population, respectively. The absence of *GSTM1* also called *GSTM1*-null was observed in 25.48% (265/1040) of which 25.79% (147/570) were in the controls and 25.11% (118/470) were in positive cases for HR-HPV (OR = 0.96; 95% CI = 0.72 - 1.27; p= 0.83). That of the *GSTT1* also called *GSTT1*-null is 44.13% with 45.79% in controls and 42.13% in positive cases for HR-HPV (OR = 0.86; 95% CI = 0.67 - 1.10; p= 0.2).

In the *GSTM1* and *GSTT1* associations, we found 40.87% (425/1040) of *GSTM1*-active/*GSTT1*-active, 10.67% of *GSTM1*-null/*GSTT1*-null (OR = 0.96; 95% CI = 0.63 - 1.46; p= 0.91); 14.71% and 33.75% of *GSTM1*-null/*GSTT1*-active (OR = 0.75; 95% CI = 0.52 - 1.10; p= 0.15) and *GSTM1*-active/*GSTT1*-null (OR = 0.75; 95% CI = 0.56 - 0.99; p= 0.05) respectively in the population. The analysis had shown in the general population non-significant associations of glutathione-S-transferase deletion and HR-HPV infection (Table 3).

On the other hand, statistically significant associations in our population was observed in carrying out of the *GSTM1*-null/*GSTT1*-active genotype with a 2.32-fold higher risk in HPV 18 infection compared to *GSTM1*-active/ *GSTT1*-active in the study population (95% CI = 1.06 - 5.08; p= 0.042). Carriers of the *GSTT1*-null genotype had a 1.72-fold higher risk of HPV 66 infection compared to other HPV genotypes (CI = 1.02 - 2.90; p= 0.044). However, there was a decrease in HPV 35 infection for women with the *GSTT1*-null genotype in the study population (OR = 0.39; 95% CI = 0.19 - 0.78; p= 0.008) (Table 5).

GSTM1 and GSTT1 polymorphism and HR-HPV infection in different countries of study

Table 4 reveals the distribution of the deletion polymorphisms of *GSTM1* and *GSTT1* in the different countries of our study. *GSTM1*-active and *GSTT1*-active were 77.75 and 62.15% respectively in Côte d'Ivoire,

Table 1. Sociodemographic data of cases and controls.

Variable	HR-HPV, n(%)	Controls, n(%)	Total, n (%)	p-value
Total	482 (100,00)	587 (100,00)	1069 (100.00)	
Mean age	35.25 ± 10.03	35.66 ± 9.2	35.48 ± 9.6	
Age groups (years)				0.077
≤ 24	60 (12.45)	64 (10.90)	124 (11.60)	
25-34	185 (38.38)	203 (34.58)	388 (36.30)	
35-44	162 (33.61)	217 (36.97)	379 (35.45)	
45-54	52 (10.79)	85 (14.48)	137 (12.82)	
≥ 55	23 (4.77)	16 (2.73)	39 (3.65)	
Unknown	(0.00)	2 (0.34)	2 (0.19)	
Marital status				0.000
Single	152 (31.54)	116 (19.76)	268 (25.07)	
Divorced	1 (0.21)	2 (0.34)	3 (0.28)	
married	315 (65.35)	451 (76.83)	766 (71.66)	
Widow	13 (2.70)	15 (2.56)	28 (2.62)	
Unknown	1 (0.21)	3 (0.51)	4 (0.37)	
Age of 1st intercourse (years)				0.000
< 15	22 (4.56)	29 (4.94)	51 (4.77)	
≥ 25	15 (3.11)	22 (3.75)	37 (3.46)	
15 ≤ X ≤ 24	407 (84.44)	435 (74.11)	842 (78.77)	
Unknown	38 (7.88)	101 (17.21)	139 (13.00)	
Number of partner				0.880
0	6 (1.24)	9 (1.53)	15 (1.40)	
1	419 (86.93)	514 (87.56)	933 (87.28)	
≥ 2	53 (11.00)	61 (10.39)	114 (10.66)	
Unknown	4 (0.83)	3 (0.51)	7 (0.65)	
Use of contraception				0.351
No	238 (49.38)	278 (47.36)	516 (48.27)	
Yes	136 (28.22)	174 (29.64)	310 (29.00)	
Unknown	108 (22.41)	135 (23.00)	243 (22.73)	
Sexual infection				0.894
No	61 (12.66)	79 (13.46)	140 (13.10)	
Yes	66 (13.69)	83 (14.14)	149 (13.94)	
Unknown	355 (73.65)	425(72.40)	780 (72.97)	
HIV infection				
Negative	116 (24.07)	125 (21.29)	241 (22.54)	
Positive	5 (1.04)	4 (0.68)	9 (0.84)	0.633
Inconnu	361 (74.90)	459 (78.02)	819 (76.61)	
VIA/VILI				
Negativef/Negative	378 (78.42)	543 (92.50)	921 (86.16)	
Negative/Positive	5 (1.04)	5 (0.85)	10 (0.94)	
Positive/Negative	7 (1.45)	6 (1.02)	13 (1.22)	0.000
Positive/Positive	91 (18.88)	29 (4.94)	120 (11.23)	
Unrealize	1 (0.21)	4 (0.68)	5 (0.47)	

Table 2. Epidemiology data of cases.

HPV type	Number (% in women infected)	Number (% in all people)
HPV 52	92 (19.09)	92 (8.61)
HPV 68	84 (17.43)	84 (7.86)
HPV 66	70 (14.52)	70 (6.55)
HPV 45	68 (14.11)	68 (6.36)
HPV 31	65 (13.49)	65 (6.08)
HPV 56	61 (12.66)	61 (5.71)
HPV 58	59 (12.24)	59 (5.52)
HPV 51	55 (11.41)	55 (5.14)
HPV 39	51 (10.58)	51 (4.77)
HPV 35	47 (9.75)	47 (4.40)
HPV 59	43 (8.92)	43 (4.02)
HPV 18	42 (8.71)	42 (3.93)
HPV 16	13 (2.70)	13 (1.22)
HPV 33	11 (2.28)	11 (1.03)
HPV- multiple infection		
No	301 (62.45)	301 (28.16)
Yes	181 (37.55)	181 (16.93)

Table 3. *GSTM1* and *GSTT1* and HR-HPV infection in West Africa.

Genotype	Controls, n (%)	HPV+, n (%)	Total, n (%)	OR (95%IC) *p*-value
GSTM1(+)	423 (74.21)	352 (74.89)	775 (74.52)	Ref
GSTM1(-)	147 (25.79)	118 (25.11)	265 (25.48)	0.96 (0.72-1.27) 0.83
GSTT1(+)	309 (54.21)	272 (57.87)	581 (55.87)	Ref
GSTT1(-)	261 (45.79)	198 (42.13)	459 (44.13)	0.86 (0.67-1.10) 0.25
GSTM1(+)/GSTT1(+)	218 (38.25)	207 (44.04)	425 (40.87)	Ref
GSTM1(-)/GSTT1(-)	58 (10.18)	53 (11.28)	111 (10,67)	0.96 (0.63-1.46) 0.91
GSTM1(-)/GSTT1(+)	89 (15.61)	64 (13.62)	153 (14.71)	0.75 (0.52-1.10) 0.15
GSTM1(+)/GSTT1(-)	205 (35.96)	146 (31.06)	351 (33.75)	0.75 (0.56-0.99) 0.05
Total	570	470	1040	

(-) Null; (+) Active; Ref: reference.

73.68 and 52.19% in Benin, 73.43 and 51.21% in Burkina Faso, 72.79 and 50.74% in Togo, and 66.67 and 56.41% in Niger. *GSTM1*-null, *GSTT1*-null, *GSTM1*-active/*GSTT1*-null, *GSTM1*-null/*GSTT1*-active, and *GSTM1*-null/*GSTT1*-null in this study did not show any significant difference between positive cases of HR-HPV and controls in the population of Burkina Faso, Togo, Benin and Niger. On the other hand, in the Ivorian population, we observed a significant difference by comparing the cases with the controls for *GSTT1*-null and the association between *GSTM1*-active/*GSTT1*-null. Their frequency in the population was respectively 37.85% (OR = 0.61; 95% CI = 0.40-0.92; p= 0.02) and 29.41% (OR = 0.56; 95% CI = 0.35-0.90; p = 0.02) with reduced risk of infection (OR <1). *GSTM1*-null in Niger, the associations *GSTM1*-null/*GSTT1*-null in Niger and in Togo had marginal risks of 2.25; 2.28 and 2.60 respectively.

GSTM1, GSTT1 polymorphisms and HPV infection in cervical lesions or dysplasia

Table 6 shows the distribution of HR-HPV infection and the deletion polymorphisms studied as a function of the results of the VIA/VILI tests. In women, HR-HPV infection was significantly associated with cervical lesions with the risk of 3.98-fold higher compared to women with negative HPV (95% CI = 2.67-5.93; p<0.0001). *GSTT1*-null had statistically significant frequencies with a reduced risk of cervical lesions compared to *GSTM1*-active (OR = 0.63; 95% CI = 0.43 - 0.91; p = 0.01). Unlike the *GSTT1*-null genotype,

Table 4. GSTM1 and GSTT1 polymorphism by country of study.

Countries	Variables	Controls [n(%)]	HR-HPV+ [n(%)]	Total [n(%)]	OR	95%IC	P-value
	GSTM1(+)	164(77.73)	140(77.78)	304(77.75)	ref		
	GSTM1(-)	47(22.27)	40(22.22)	87(22.25)	0.99	0.61-1.60	1.00
	GSTT1(+)	120(56.87)	123(68.33)	243(62.15)	ref		
	GSTT1(-)	91(43.13)	57(31.67)	148(37.85)	0.61	0.40-0.92	0.02
Cote d'Ivoire	GSTM1(+)/GSTT1(+)	92(43.60)	97(53.89)	189(48.34)	ref		
	GSTM1(-)/GSTT1(+)	28(13.27)	26(14.44)	54(13.81)	0.88	0.48-1.61	0.75
	GSTM1(+)/GSTT1(-)	72(34.12)	43(23.89)	115(29.41)	**0.56**	**0.35-0.90**	**0.02**
	GSTM1(-)/GSTT1(-)	19(9.00)	14(7.78)	33(8.44)	0.69	0.33-1.47	0.45
	Total	211(100)	180 (100)	391(100)			
	GSTM1(+)	94(72.87)	74(74.75)	168(73.68)	ref		
	GSTM1(-)	35(27.13)	25(25.25)	60(26.32)	0.90	0.49-1.64	0.76
	GSTT1(+)	67(51.94)	52(52.53)	119(52.19)	ref		
	GSTT1(-)	62(48.06)	47(47.47)	109(47.81)	0.97	0.57-1.65	1.00
Benin	GSTM1(+)/GSTT1(+)	49(37.98)	41(41.41)	90(39.47)	ref		
	GSTM1(-)/GSTT1(+)	18(13.95)	11(11.11)	29(12.72)	0.73	0.30-1.72	0.52
	GSTM1(+)/GSTT1(-)	45(34.88)	33(33.33)	78(34.21)	0.87	0.47-1.61	0.75
	GSTM1(-)/GSTT1(-)	17(13.18)	14(14.14)	31(13.60)	0.98	0.43-2.23	1.00
	Total	129 (100)	99(100)	228(100)			
	GSTM1(+)	73(67.59)	79(79.80)	152(73.43)	ref		
	GSTM1(-)	35(32.41)	20(20.20)	55(26.57)	0.52	0.27-0.99	0.058
	GSTT1(+)	57(52.78)	49(49.49)	106(51.21)	ref		
	GSTT1(-)	51(47.22)	50(50.51)	101(48.79)	1.14	0.66-1.96	0.67
Burkina Faso	GSTM1(+)/GSTT1(+)	32(29.63)	37(37.37)	69(33.33)	ref		
	GSTM1(-)/GSTT1(+)	23(21.30)	12(12.12)	35(16.91)	0.45	0.19-1.04	0.09
	GSTM1(+)/GSTT1(-)	41(37.96)	42(42.42)	83(40.10)	0.88	0.46-1.67	0.74
	GSTM1(-)/GSTT1(-)	12(11.11)	8(8.08)	20(9.66)	0.57	0.20-1.58	0.31
	Total	108(100)	99(100)	207(100)			
	GSTM1(+)	56(76.71)	43(68.25)	99(72.79)	ref		
	GSTM1(-)	17(23.29)	20(31.75)	37(27.21)	1.53	0.71-3.27	0.33
	GSTT1(+)	38(52.05)	31 (49.21)	69(50,74)	ref		
	GSTT1(-)	35(47.95)	32(50,79)	67(49.26)	1.12	0.57-2.19	0.86
Togo	GSTM1(+)/GSTT1(+)	26(35.62)	22(34.92)	48(35.29)	ref		
	GSTM1(-)/GSTT1(+)	12(16.44)	8(12.70)	20(14.71)	0.78	0.27-2.27	0.79
	GSTM1(+)/GSTT1(-)	30(41.10)	22(34.92)	52(38.24)	0.86	0.39-1.91	0.84
	GSTM1(-)/GSTT1(-)	5(6.85)	11(17.46)	16(11.76)	2.60	0.78-8.63	0.15
	Total	73(100)	63(100)	136(100)			
	GSTM1(+)	36(73.47)	16(55.17)	52(66.67)	ref		
	GSTM1(-)	13(26.53)	13(44.83)	26(33.33)	2.25	0.85-5.92	0.13
	GSTT1(+)	27(55.10)	17(58.62)	44(56.41)	ref		
	GSTT1(-)	22(44.90)	12(41.38)	34(43.59)	0.86	0.34-2.19	0.81
Niger	GSTM1(+)/GSTT1(+)	19(38.78)	10(34.48)	29(37.18)	ref		
	GSTM1(-)/GSTT1(+)	8(16.33)	7(24.14)	15(19.23)	1.66	0.46-5.92	0.52
	GSTM1(+)/GSTT1(-)	17(34.69)	6(20.69)	23(29.49)	0.67	0.20-2.23	0.56
	GSTM1(-)/GSTT1(-)	5(10.20)	6(20.69)	11(14.10)	2.28	0.55-9.36	0.29
	Total	49(100)	29(100)	78(100)			

(-) Null; (+) Active.

Table 5. Association between GSTT1, GSTM1 polymorphisms and HPV type.

Polymorphisms	Women others	HPV35 infected women	OR (95%IC) p-value	Total
GSTT1 (+)	547	34	ref	581
GSTT1 (-)	448	11	0.39 (0.19-0.78) **0.008**	459
Total	995	45		1040
	Women others	**HPV18 infected women**	**OR (95%IC) p-value**	**Total**
GSTM1(+)/GSTT1(+)	410	15	ref	425
GSTM1(+)/GSTT1(-)	337	14	1.13 (0.54-2.38) 0.849	351
GSTM1(-)/GSTT1(+)	141	12	2.32 (1.06-5.08) **0.042**	153
GSTM1(-)/GSTT1(-)	110	1	0.2 (0.03-1.90) 0.213	111
Total	998	42		1040
	HPV others	**HPV66 infected women**	**OR (95%IC) p-value**	**Total**
GSTT1(+)	241	31	ref	272
GSTT1(-)	162	36	1.72 (1.02-2.90) **0.044**	198
Total	403	67		470

Table 6. Association between HPV infection, GSTM1, GSTT1 polymorphisms and VIA/VILI results.

Variable		*VIA/VILI*				Total
		Unrealized	Without dysplasia	With dysplasia	OR (95%IC) p-value	
HPV infection	Negative	3	530	37		570
	Positive	1	367	102	3.98 (2.67-5.93) **<0.0001**	470
GSTM1	Active	3	672	100		775
	Null	1	225	39	1.16 (0.78-1.73) 0.46	265
GSTT1	Active	1	489	91		581
	Null	3	408	48	0.63 (0.43-0.91) **0.01**	459
Total		4	897	139		1040

Table 7. IVA/IVL, *GSTM1*, *GSTT1* and HPV infection.

		GSTM1			GSTT1			
	IVA/IVL	**Null**	**Active**	**OR (95%IC) p-value**	**Null**	**Active**	**OR (95%IC) *p*-value**	**Total**
HPV-	Unrealized	1	2		2	1		3
	Without dysplasia	134	396		243	287		530
	With dysplasia	12	25	1.41 (0.69-2.90) 0.33	16	21	0.89 (0.45-1.76) 0.86	37
	Total	147	423		261	309		570
HPV+	Unrealized	0	1		1	0		1
	Without dysplasia	91	276		165	202		367
	With dysplasia	27	75	1.09 (0.66-1.79) 0.79	32	70	0.55 (0.35-0.89) **0.01**	102
	Total	118	352		198	272		470

GSTM1-null and the double null genotype did not have statistically significant results. However, a risk of 0.55 was found in the double genotype *GSTM1*-active/*GSTT1*-null compared to *GSTM1*-active/*GSTT1*-active genotype in cervical lesions (95% CI = 0.35-0.86; p = 0.009). Table 7 presents the HPV, GST statuses as a function of the test results; women carrying *GSTT1*-null genotype, and HPV positive had a statistically significant result. Cervical lesions are linked to reduction in risk compared to *GSTT1*-active (OR = 0.55; 95% CI = 0.35-0.89; p = 0.01).

GSTM1, GSTT1 deletion and socio-demographic characteristic

The study did not show significant results of the respondents' socio-demographic characteristic such as age, marital status, age of 1st intercourse, contraception using, sexual partner, sexual infection, HIV infection with deletion of *GSTM1* and *GSTT1*.

DISCUSSION

The present study aims to assess the risk of *Glutathione S-Transferase M1* and *T1* polymorphisms in HR-HPV infection and cervical lesions or dysplasia in women. In literature, very few studies have been carried out on HR-HPV infection in relation to the deletions of *GSTM1* and *GSTT1*. Our study is the first in sub-Saharan Africa and West Africa particularly, where HPV infection is very common.

In this study, 29 samples (02.71%) were invalid after PCR testing. Different studies on polymorphisms have largely used blood samples (Ueda et al., 2010; Agodi et al., 2010). In our study, we used endocervical cells samples. There were very few samples, with a low DNA concentration when checked with the nanodrop. Endocervical cells could explain this low DNA concentration after extraction. This would therefore lead to illegible bands after electrophoresis.

Oxidative stress, according to studies, had been associated with an increase in viral replication (Scholz et al., 1996; Koike, 2009). According to De Marco et al. (2012), increased oxidants are associated with the neoplastic progression of HPV16 (De Marco et al., 2012). Several factors are responsible for oxidative stress. The genetic factors are the most crucial and influential. The main enzyme P450 cytochromes, catalyze the different oxidation reactions in phase I, producing oxidative stress by the metabolic activation of chemical carcinogens and xenobiotics (Lang and Pelkonen, 1999). The products resulting from this phase are reactive electrophilic intermediates and can cause lipids, proteins and DNA damage (Shackelford et al., 2000). Glutathione S-transferases, one of the groups of phase II enzyme, neutralize these reactive electrophiles by conjugating them with glutathione, making them more soluble in water (Ketterer et al., 1993; Wilce and Parker, 1994; Armstrong, 1997), and thus eliminating oxidative stress. The deletions of genes by these enzymes could lead to the persistence of oxidative stress, causing viral multiplication, DNA damage by reactive electrophiles attacks or chromosomal instability, and consequently the carcinogenic process.

According to Ueda et al. (2010) certain high-risk HPV-infections were associated with polymorphisms of glutathione S-transferase. Our study did not show an association between the *GSTM1*-null, *GSTT1*-null genotypes and HR-HPV infection in West Africa population. These results are similar to those found by Agodi et al. (2010) in Italy. Their results and ours suggested that the deletion of *GSTM1*, *GSTT1* genotypes is not associated in general with HPV-infection. However, particularities could exist according to the HR-HPV types.

The study also showed that the double *GSTM1*-active/*GSTT1*-null genotype was associated with HR-HPV infection, particularly in Côte d'Ivoire, with a relative risk always <1 (OR = 0.56). These results, although significant, showed no increased risk of HR-HPV infection, but rather a decrease in risk. The double *GSTM1*-active/*GSTT1*-null genotype could protect women from HPV-infection. *GST*s could modulate signal transduction pathways involved in cell survival and apoptosis, by controlling the activity of protein members of the mitogen-activated kinase family (MAPK) (Laborde, 2010; Singh, 2015). In normal conditions, ASK1 would be sequestered by *GSTM1* enzyme and forming the *GSTM1*/ASK1 complex. In oxidative stress conditions or heat shock, there is dissociation of the complex and activation of ASK1 and subsequently the induction of the apoptosis process. It could contribute to kill cell-infection although GSTT1 is an exposure factor.

In addition, in the general population of study, by comparing each type of HR-HPV in the study population and each type of HR-HPV with the other remaining HR-HPV, there is an association with increased risk between infection with HPV18 and double genotype *GSTM1*-null/*GSTT1*-active (OR = 2.32; p = 0.042). Agodi et al. (2010), as in this study, did not find a significant association between the suppression of the *GSTM1*, *GSTT1* genes and infection with HPV16 despite the fact that this is considered the most common in premalignant and malignant cervical lesions (Muñoz et al., 2003). The study also showed an association between HPV66 infection and the *GSTT1*-null genotype with an increased risk (OR = 1.72; p= 0.04) and another association between HPV35 infection and the *GSTT1*-null genotype (OR = 0.39; p= 0.008) with risk reduced. These results differ from those found by Ueda et al.(2010) in Japan who reported a significant association between HPV16/18 infection and the *GSTT1*-null genotype (p= 0.029) compared to the other HR-HPV. However, we have not found in the literature an association between HPV66 and HPV35 infection with *GSTT1*-null genotype. This could be explained by the very few number of studies on HR-HPV infection and polymorphism of glutathione S-transferase. This peculiarity of HPV66, HPV35 association with *GSTT1*-null in West Africa could also be due to the diversity of emergence of other high-risk HR-HPV (than HPV16/18), which are more frequent in these countries (Zohoncon et al., 2020).

In literature according to several studies and reviews, *GSTT1*-null and *GSTM1*-null could be associated with the development of cervical cancer (Lee et al., 2004; Ueda et al., 2005; Ueda et al., 2010; Wang et al., 2011; Liu and

Xu, 2012; Sun and Song, 2016; Liu et al., 2017). However, our data were collected by questionnaires and the visual inspection tests were done with acetic acid and lugol (VIA/VILI) for the detection of cervical lesions or dysplasia in the endocervix. Lack of confirmations of the lesions types and grade by biopsy after the VIA/VILI tests constitutes a limitation of the study. Although the IVA/IVL screening tests recommended for low-resource countries are necessary, the results are not very specific like those of histology or cytology.

Regarding the deletions of *GSTM1* and *GSTT1* genes and the cervical lesions, there is an association between *GSTT1*-null genotype and cervical lesions by VIA/VILI detection (p= 0.01) with a decrease in risk (OR = 0.63). Satinder et al. (2017) in India, also found a reduced risk linked to cervical cancer (OR = 0.5; p= 0.04) in carriers of the *GSTT1*-null genotype. The GSTs, according to certain studies do not lead to detoxification complete (van Bladeren, 2000). The conjugate could be reversible or not stable and could lead to high toxic metabolite. It was demonstrated that the GST-dependant conjugation involving GSTT1-null enzyme for certain substrate such as hydrocarbon mono- or di-halogens would lead to very reactive electrophile responsible for carcinogenesis (Hayes and Pulford, 1995, van Bladeren, 2000). In view of these results, the *GSTT1*-null genotype could contribute to a reduction in the risk of acquiring lesions and cancer of the cervix.

Our results showed an absence of association between *GSTM1*-null and cervical lesions (p= 0.46). Several studies have shown this absence in association between the *GSTM1*-null genotype and the intraepithelial lesions in Italy (Agodi et al., 2010; Palma et al., 2010), India (p= 0.67) (Sharma et al., 2015), and Serbia (p= 0.07) (Stosic et al., 2014). An absence of *GSTM1*-null association was also noted in India in Squamous cell carcinomas (SCC) and adenocarcinoma (AC) (Abbas et al., 2013; Satinder et al., 2017) and in cervical cancer in Turkey (p= 0.73) (Kiran et al., 2010). In addition, in women with SCC and AC in Brazil, Tacca et al. (2018) determined survival of 80.0% in women with *GSTM1*-active and 73.3% in women with *GSTM1*-null after a 60-month follow-up and also found statistically insignificant results (p= 0.368). These results suggested that the genotype *GSTM1*-null could not be associated with lesions and cervical cancer.

In terms of gene-gene interactions, the study showed a reduction in the risk of cervical lesions (OR = 0.55; p = 0.009) for carriers of the *GSTM1*-active / *GSTT1*-null double genotype. Furthermore, Satinder et al. (2017) found an OR <1 also statistically significant in India for the double genotype *GSTM1*-active / *GSTT1*-null for cervical cancer (OR = 0.4; p= 0.02) and SCC (OR = 0.4; p= 0.04). This is contrary to our study which focused on cervical lesions by IVA/IVL detection. The double *GSTM1*-active/*GSTT1*-null genotype would contribute, just like the *GSTT1*-null genotype to reduce the risk of cervical lesions according to our study. *GSTT1* and *GSTM1* could play an important role in oxidative stress or in the metabolization of xenobiotic or regulation cell, which could partially compensate for the absence of one of two.

The enzymes responsible for the metabolism of carcinogens would be important risk modifiers in carcinogenesis (Sheweita, 2000). Thus, in the case of persistent infection with HR-HPV, oxidative damage to DNA caused by enzyme deletion could indeed serve as a mechanism to facilitate the integration of HPV, and subsequently, carcinogenesis (Williams et al., 2011). In the study, in women infected with HR-HPV, there were no statistically significant results (p= 0.79) in cervical lesions by VIA/VILI detection in carriers of *GSTM1*-null genotype. Nunobiki et al. (2015) also showed statistically insignificant results (p= 0.35) of the *GSTM1*-null genotype in intraepithelial lesions de haut grade (HSIL) in Japan among women infected with HPV. The mean results of the *GSTM1*-null genotype could not contribute to cervical lesions in women with HPV-infection.

GSTT1-null genotype had statistically significant results (OR = 0.55; p= 0.01) in women with HR-HPV+ and its risk could be reduced for cervical lesions. However in Japan (Nunobiki et al., 2015), Hungary (Cseh et al., 2011) and India (Joseph et al., 2006), they found an increased risk. In the studies by Nunobiki et al. (2015) *GSTT1*-null was significantly associated with low grade to high grade lesions in HR-HPV positive patients in Japan (OR = 3.45). According to the studies of Cseh et al. in Hungary after 7 years following women with HPV positive, a risk of 1.89-fold higher was attributed to the development of HSIL compared to controls. Joseph et al. determined in India a 19.25-fold higher risk of acquiring invasive cancer and HSIL in women with *GSTT1*-null genotype, and HPV16 positive compared to controls with normal cytology and low-grade lesions.

As for the double null genotype, we did not find significant results in West Africa for HPV infection and cervical lesions by VIA/VILI detection. In India, Romania and Serbia, non-significant results for the interactions of both null alleles were demonstrated (Stosic et al., 2014; Sharma et al., 2015; Daniel et al., 2016). However GSTs role was demonstrated in cells (Laborde, 2010; Gao et al., 2011; De Marco et al., 2012; Singh, 2015; Wang et al., 2016). Cseh et al. (2011) in Hungary after 7 years of follow up determined a relative risk of 2.35 of HSIL development for women infected with HR-HPV carrying a double null genotype compared to those who have at least one genotype present. Environmental factors could more or less contribute as a cofactor to cervical lesions. It would be more interesting to study the environmental factors as a source of xenobiotic contribution in detoxification genes deletions relation, which could play an important role.

The different case/control studies on the deletions of *GSTM1* and *GSTT1* by other authors, in addition to performing biopsies unlike ours, included the action of

passive and active smoking and exposure to wood smoke (Palma et al., 2010; Abbas et al., 2013; Stosic et al., 2014; Sharma et al., 2015; Satinder et al., 2017; Tacca et al., 2018). Thus, these authors determined statistically significant results between cancer and smoking (Abbas et al., 2013; Tacca et al., 2018); exposure to wood smoke (Satinder et al., 2017), and between cancer, smoking, and *GSTM1* and *GSTT1* polymorphisms (Sobti et al., 2006; Sharma et al., 2015). In this study, socio-demographic characteristics of the respondents such as smoking and exposure to wood smoke were not included. Two other authors carried out a study on a patient followed-up in Hungary and Brazil (Cseh et al., 2011; Tacca et al., 2018). The size of our samples was disproportionate between the number of women positive for at least one of the positive VIA/VILI tests and those with negative VIA/VILI (139/897), which also does not allow analysis by country.

Conclusion

Our results suggest a reduction in the progression of high-risk HPV infection in the Ivory Coast Republic, in carriers of *GSTT1*-null and of the double *GSTM1*-active/*GSTT1*-null in women with HR-HPV infected. Among women with HR-HPV infection, *GSTT1*-null genotype could reduce the risk of progression of HPV35 infection and increase HPV66 infection in West Africa. The risk of progression of HPV18 infection would be favored by the *GSTM1*-null/*GSTT1*-active genotype in HR-HPV positive women from West Africa. The risk reduction in the acquisition or progression of cervical lesions by VIA/VILI detection could be justified by carrying out the *GSTT1*-null genotype in the study population and women with HPV infection. *GSTM1*-active/*GSTT1*-null may be associated with the reduction of precancerous lesions in West Africa. A study including other genetic cofactors, environmental and confirmed lesions and cancer, would be necessary to shed more light on the various factors influencing carcinogens of cervical cancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular characterization of viruses causing maize lethal necrosis disease in South-Rift region, Kenya

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The first report of Maize lethal necrosis (MLN) disease in Africa was in Bomet, Kenya, nine years ago. It has since spread in East and Central African (ECA) countries, causing massive yield losses. Currently, 90% of the preferred commercial maize germplasm grown by farmers in Kenya is susceptible to MLN disease. As such, the disease has continued to pose a serious challenge to food security in the ECA region. This study sought to characterize the MLN, causing viruses present in the maize leaf samples collected from the South-Rift region. Using total RNA extracted from 60 leaf samples collected from Bomet, Kericho, and Kisumu Counties, reverse transcription-polymerase chain reaction (RT-PCR) was carried out. The PCR products with the strongest bands were purified and sequenced using the Sanger sequencing technique. The results showed that samples from the three counties were positive for maize chlorotic mottle virus (MCMV) (MH645622 and MH645621) and sugarcane mosaic virus (SCMV) (MH645623, MH645624 and MH645625) and negative to wheat streak mosaic virus (WSMV). The coat protein (CP) sequences of MCMV isolates were closely related to the sequences of MCMV isolates, which had been previously reported from Eastern and Sub-Saharan Africa. For the CP sequences of SCMV isolates, only one sequence of the isolate KCO59 was similar to the sequence of a Kenyan isolate (JX286708). Sequences of isolates KCO5 and KCO24 were not identical to those of the Kenyan isolate (JX286708). Based on these results, in the surveyed counties, isolates of SCMV are genetically diverse, while those of MCMV are not. There exists a new variant of SCMV, which appears to be the main potyvirus in synergism with MCMV in causing MLN disease in Kenya.

Key words: Maize lethal necrosis (MLN), maize chlorotic mottle virus (MCMV), sugarcane mosaic virus (SCMV), wheat streak mosaic virus (WSMV).

INTRODUCTION

Maize lethal necrosis (MLN) disease has emerged as one of the major threats to food security in Sub-Saharan Africa as maize is a staple food crop (Mbega et al., 2016). The disease has become a significant concern in

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Table 1. Primers used in cDNA synthesis.

Virus	Sequence name	Sequence	Amplicon (bp)
MCMV	MCMV For.	TGGCATTCCTCTCCGATTAC	122
	MCMV Rev.	GTTCTGTGGCATGACAATGG	122
SCMV	SCMV For.	TATGGACGGAGACGAACAAAG	186
	SCMV Rev.	TCTTGGCATGTATCGCTCTG	186
WSMV	WSMV For.	AACAGAGCACTCGGGACTTG	195
	WSMV Rev	TGAAACTGTGCGTGTTCTCC	195

Eastern Africa, having been first reported in Bomet County in the South-Rift region of Kenya in 2011 (Wangai et al., 2012). Since then, it has spread to other maize-growing areas in Central, Nyanza, Western, South, and North-Rift regions of Kenya (Davis, 2012). The disease has also been reported in other countries in East Africa where maize is an outstanding staple food. These countries include; Rwanda (Adams et al., 2014), Democratic Republic of Congo (DRC) (Lukanda et al., 2014), Tanzania (Kiruwa et al., 2016) Uganda (Kagoda et al., 2016) and more recently from South Sudan and Ethiopia (Mahuku et al., 2015). Yield losses as high as 59% or more than 300,000 tons have been reported in moist transitional zones, mostly in Western Kenya (De Groote et al., 2016) due to MLN disease. The disease has been reported to be a result of synergistic interaction between *Maize chlorotic mottle virus* (MCMV) with any potyvirus such as *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV) or *Wheat streak mosaic virus* (WSMV) (Isabirye and Rwomushana, 2016). The synergistic interactions have been reported to be more pronounced and result in severe damage that usually kills the infected plant (Makone et al., 2014). Due to such magnitude of loss posed by the disease, it has discouraged some farmers from planting maize in the second season (Kamau and Snipes, 2014), a situation that is likely to affect maize production in the region.

Though there are commercial varieties that have already been released to farmers with appreciable tolerance to MLN, most farmers have little knowledge of how the disease spread and its control measures (Mahuku et al., 2015). Due to such inadequacies, most of the farmers practice continuous maize cropping, a practice that has been reported as one of the factors contributing to the build-up of the virus inoculum (Mezzalama, 2015). With this in mind, there is a need to characterize the virus isolates from the South-Rift region to identify the virus causing MLN disease in this region and assess their diversity. Therefore, this study sought to isolate viruses from the maize leaf samples collected from the South-Rift region in Kenya and sequence their coat protein gene.

MATERIALS AND METHODS

Sample collection

Maize leaf samples were collected between July and August 2016 in three counties of the South-Rift region of Kenya; Bomet, Kericho, and Kisumu. These counties were selected as MLN occurrence was first reported in September 2011 in Longisa in Bomet (Wangai et al., 2012), which is in Kenya's South-Rift region. Random sampling was used to select the maize farms and sampling sites. Ten farms were randomly selected from each county, and in each farm, five samples were collected. Sampling points within each maize farm were restricted to symptomatic plants, and the upper young leaf from maize plants showing MLN disease symptoms was collected. The collected leaf samples were stored in Ziploc paper storage bags in a cool box, labeled with the maize variety, where the isolate was obtained from, and the county. They were shipped to Botany and Plant pathology laboratory at Purdue University in USA for isolation of RNA and sequencing.

Extraction of RNA

From the 150 maize samples collected, 20 samples were randomly selected from each county for RNA isolation. The total RNA was extracted from individual maize leaves using the Trizol reagent (Invitrogen) as described by Chomzynski and Mackey (1995) followed by the RNeasy (QIAGEN) method as described by Adams et al. (2012) at Purdue University, Botany and Plant pathology laboratory, USA. The quantity of RNA and its purity was determined using the NanoDrop machine (2000c model) and stored at -80°C until the RT-PCR was done.

Reverse transcription (RT)-PCR amplification

RT-PCR amplification was carried out according to Jarugula et al. (2010) with modifications. It was a two-step RT-PCR reaction where cDNA was prepared from 1 µl of RNA using a Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Wilmington, DE) as per the instruction manual. The cDNA primers (Table 1) were designed using the qPCR Primer Quest tool, Integrated DNA Technologies Inc.

The specific primers for amplification of the CP gene were designed based on the reported sequence of MCMV (JX286709) and SCMV (Adams et al., 2012) (Table 2). The RT-PCR was carried out using a thermal cycler (GeneAmp PCR systems 9700, Applied Biosystems) to amplify CP-specific DNA fragments. The cycling conditions were one cycle at 95°C for 5 min to allow denaturation, followed by 35 cycles at 94°C for 30s, 58°C for 30 s,

Table 2. Primers used to amplify the CP of MCMV, SCMV, and WSMV variants.

Virus	Sequence name	Sequence	Gene	Amplicon (bp)
MCMV	MCMV For.	TGGAAAACATTGCTGTTGGA	CP	1066
	MCMV Rev.	CAGGACTCTGCCAGAAGGAC	,,	1066
SCMV	SCMV For.	GCACAGGGATCAAGGAAGAA	,,	1192
	SCMV Rev.	TGTCCTGCAGACTGGTTCAC	,,	1192
WSMV	WSMV For.	ATGGCCAGATTTCAAGTTGC	,,	1275
	WSMV Rev.	TGTACGCCTCTCGTGAAGAA	,,	1275

72°C for 60 s; and a final extension at 72°C for 7 min. An aliquot of the RT-PCR assay product was resolved on 1.5% agarose gels, stained with ethidium bromide, and the DNA bands were visualized under a UV-transilluminator.

Sequencing of the CP gene of MCMV and SCMV variants

Polymerase chain reaction products from the samples with the strongest bands when viewed under the U.V. light were purified for sequencing using the Qiagen kit following the manufacturer's instructions (QIAGEN Inc., Valencia, CA). The eluted DNA from MCMV and SCMV was used for sequencing, while WSMV was not sequenced since all the samples tested negative. Sequencing was done at Purdue Genomics Core Facility, USA, using the Sanger sequencing technique. The resulting sequences were then compared to published sequences in the GenBank-NCBI database (Benson et al., 2013) to give the identities of the sequences using blast+ (Camacho et al., 2009).

Sequence alignment and construction of phylogenetic trees

Multiple nucleotide sequence alignments were performed using Clustal Omega version 7. Manual editing was done to obtain consensus sequences. Aligned MCMV and SCMV sequences were assessed using DnaSP version 5.1 (83) software to estimate genetic diversity, and phylogenetic trees were constructed using the MEGA program version 7 (Kumar et al., 2016).

RESULTS

Maize lethal necrosis (MLN) symptoms observed in the field

As shown in Figure 1, the MLN symptoms illustrate the extent of the damage the disease poses on maize plants, especially among the susceptible varieties viz a viz the clean, uninfected maize plant. Maize plants in picture **A** show excessive chlorotic mottling with short internodes, and necrosis on leaf margin that progresses towards the midrib; while in picture **B**, the maize plants appear dwarf with severe chlorotic mottling on the upper leaves. The lower leaves have dried up due to excessive necrosis resulting in premature aging of the plants. As for picture **C**, the maize plants show no MLN symptoms.

RT-PCR detection of viruses causing MLN disease

The DNA from the 60 samples was amplified in the RT-PCR reaction. Out of these, 20 samples were positive to MCMV, while 18 samples were positive to SCMV. As for WSMV, all the 60 samples amplified were negative (Table 3).

Coat protein (CP) gene sequence of MCMV

The CP sequence of MCMV isolates KCO 24 (accession # MH645622) and KSM 5 (accession #MH645621) were 100% identical to each other (Table 4). Also, the two isolates had 100% identity with Sub-Saharan isolate (KP851970), Ethiopia isolate (KP772217), and 100% identical to Rwanda isolate (KF744393). The sequences also had 99.86% identity to one isolate previously reported from Kenya (JX286709), 99% identity with MCMV isolates from Thailand (AM490791), and China (GU594293). Similarly, the sequences of the MCMV from the two Counties had 96.62% identity with sequences of MCMV isolates from Kansas (KS1) and Nebraska (EU358605) in the United States (Figure 2).

Coat protein (CP) gene sequence of SCMV

The KCO 5 (accession #MH645623) and KCO 24 SCMV (accession #MH645624) isolates had 99% identity with each other (Table 5), and 97% identity to KCO 59 (accession #MH645625) isolate and Kenya isolate (JX28708). Similarly, the SCMV isolate KCO 59 had 98.29% identity with the Kenya isolate (JX28708). The three SCMV isolates KCO5, KCO24, and KCO 59 had a similarity ranging from 76.97 to 81.24% with the other SCMV isolates from India, Pakistan, Australia, China, and Argentina (Figure 3).

DISCUSSION

The objective of the study is to characterize the viruses

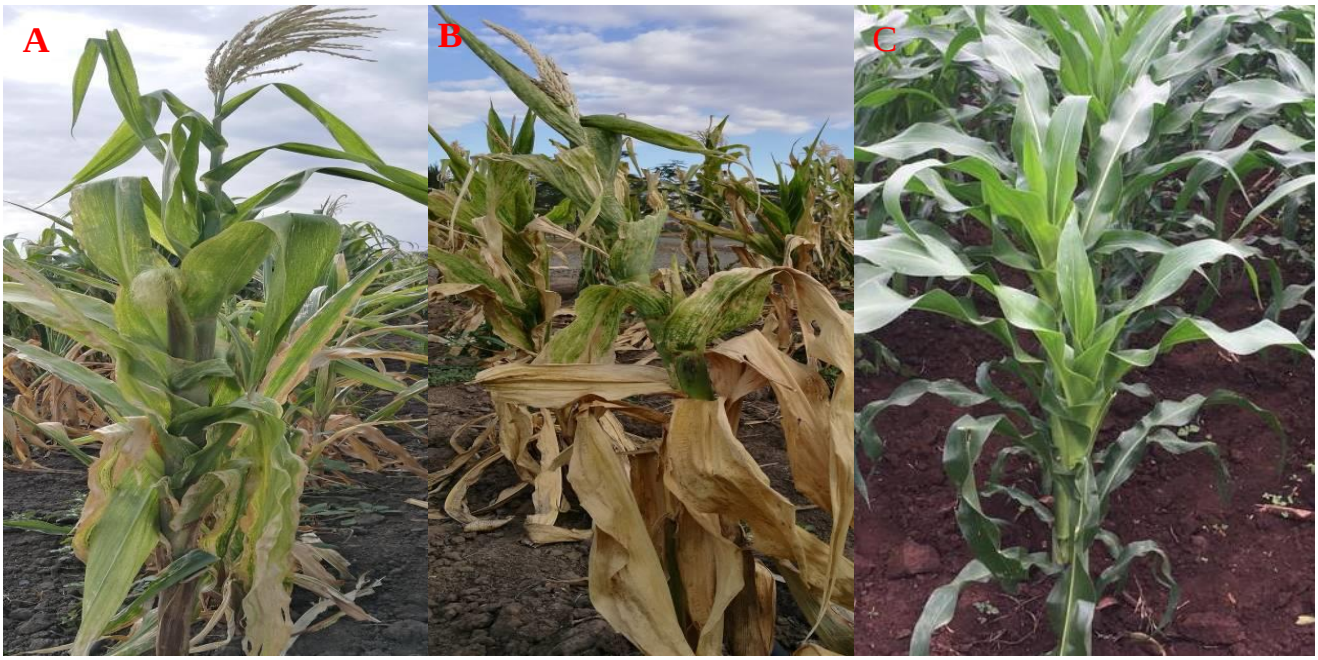

Figure 1. MLN symptoms on Maize plant. **A**-Severe chlorosis; **B**-Complete necrosis on the lower leaves, **C**-Uninfected maize plant. The pictures were taken in the field during the survey.

Table 3. RT-PCR detection of MCMV, SCMV, and WSMV.

Virus	No. of samples tested	Positive (+ve)	Positive (%)
MCMV	60	20	33
SCMV	60	18	30
WSMV	60	0	0

Table 4. Percentage identities between MCMV isolates from this study and isolates from the GenBank-NCBI.

Sequence	Accession	KS	NEB	KSM	KCO	SSA	ETP	RW	KEN	TH	C.N.
Kansas	KS1	100	99.58	96.62	96.62	96.62	96.62	96.62	96.48	96.77	97.05
Nebraska	EU358605	99.58	100	96.34	96.34	96.34	96.34	96.34	96.2	96.48	97.05
Kisumu	KSM5	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Kericho	KCO24	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
SSA	KP851970	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Ethiopia	KP772217	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Rwanda	KF744393	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Kenya	JX286709	96.48	96.2	99.86	99.86	99.86	99.86	99.86	100	98.87	98.87
Thailand	AM490791	96.77	96.48	99.02	99.02	99.02	99.02	99.02	98.87	100	99.44
China	GU594293	97.05	97.05	99.02	99.02	99.02	99.02	99.02	98.87	99.44	100

KS: Kansas, **NEB**: Nebraska, **KSM**: Kisumu, **KCO**: Kericho, **SSA**: Sub-Saharan Africa, **ETP**: Ethiopia, **R**.W.: Rwanda, **KEN**: Kenya, **TH**: Thailand, **C**.N.: China

causing MLN in the South-Rift region of Kenya. From the results, only two viruses except for WSMV were detected in the samples from all the three counties studied. As reviewed in Mbega et al. (2016), MLN disease is caused by synergistic co-infection of maize MCMV with any cereal virus in the potyvirus MDMV, WSMV, or SCMV.

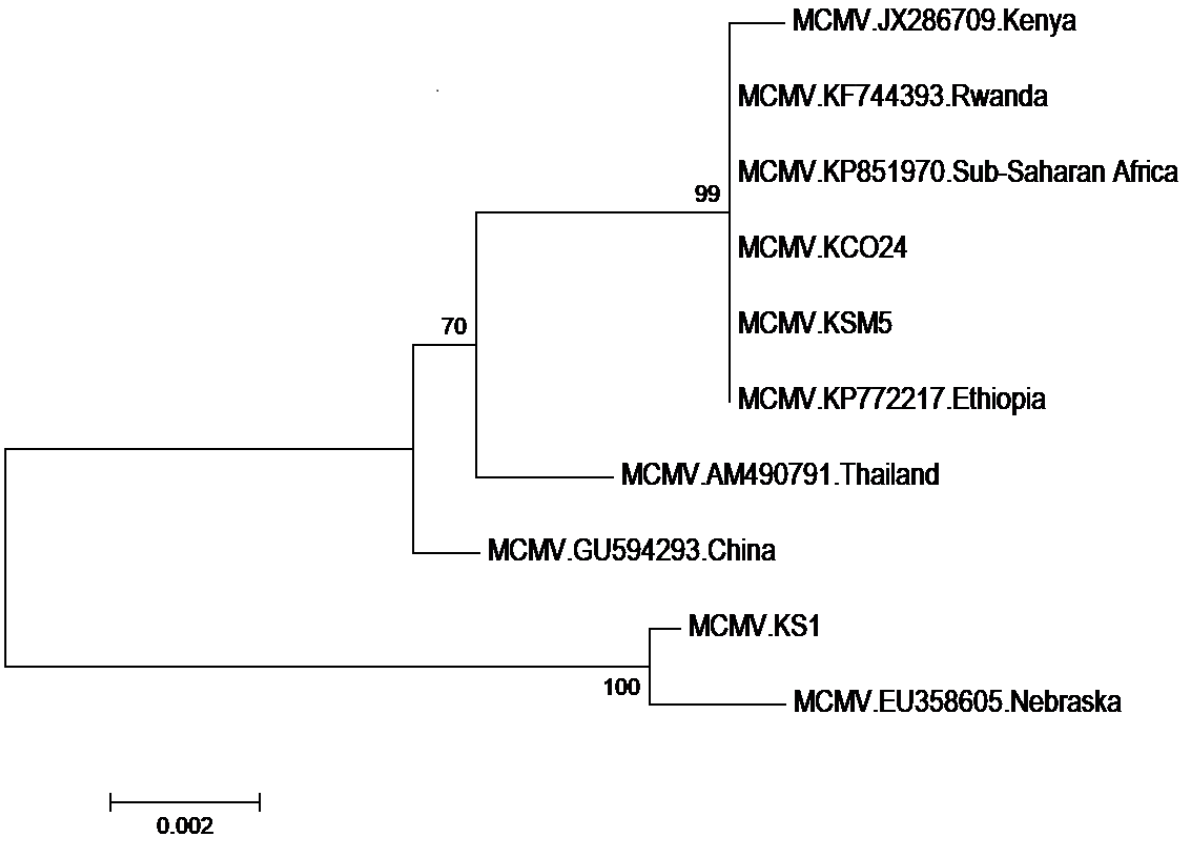

Figure 2. Phylogenetic relationships between the two isolates of MCMV coat protein sequences with other MCMV accessions from NCBI. The phylogeny was based on 711 aligned nucleotide bases. The evolutionary history with MEGA version 7, maximum likelihood method (Kumar et al., 2016) with associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 5. Percentage identities between SCMV isolates from this study and isolates from the GenBank-NCBI.

Sequence	Accession	IND	PAK	AUS	KCO5	KCO24	KCO59	KEN	CN	ARG
India	DQ866744	100	93.9	96.73	78.77	79.34	79.11	78.91	78.95	78
Pakistan	KU557299	93.69	100	96.3	76.97	77.3	77.08	77.33	79.08	78.2
Australia	AF006735	96.73	96.3	100	78.86	79.18	79.08	78.78	80.02	79.08
Kericho	KCO5	78.77	76.97	78.86	100	99.15	97.01	97.01	81.13	80.47
Kericho	KCO24	79.34	77.3	79.18	99.15	100	97.23	97.01	81.24	80.36
Kericho	KCO59	79.11	77.08	79.08	97.01	97.23	100	98.29	80.92	80.47
Kenya	JX286708	78.91	77.33	78.78	97.01	97.01	98.29	100	81.04	80.92
China	AY660663-1	78.95	79.08	80.02	81.13	81.24	80.92	81.04	100	93.18
Argentina	JX237958	78	78.2	79.08	80.47	80.36	80.47	80.92	93.18	100

IND: Indian, **PAK**: Pakistan, **AUS**: Australia, **KCO**: Kericho, **Ken**: Kenya, **CN**: China, **ARG**: Argentina.

This study's findings have demonstrated that SCMV is the main potyvirus that causes the MLN disease in the South-Rift region. These findings concur with Adams et al. (2014), who also reported that SCMV is the most frequent potyvirus in Kenya and East Africa. Comparison of CP sequences of MCMV isolates (accession # MH645622 & #MH645621) from this study with other CP sequences of MCMV available in the GenBank-NCBI shows that they are closely related to Sub-Saharan Africa isolate (KP851970), Ethiopia isolates (KP772217), Rwanda isolates (KF744393), and Kenya isolate (JX286709). This high degree of MCMV sequence

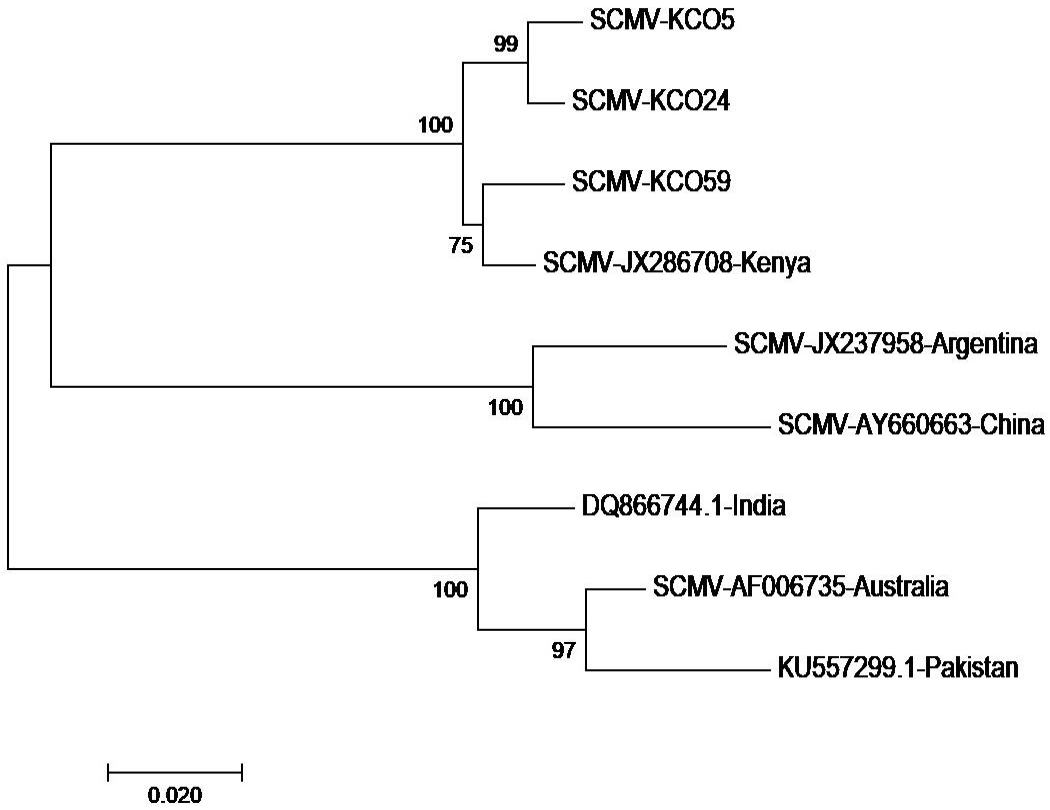

Figure 3. Phylogenetic relationships among three isolates of SCMV coat protein sequences with other SCMV accessions from NCBI. The phylogeny was based on 950 aligned nucleotide bases. The evolutionary history with MEGA version 7, maximum likelihood method (Kumar et al., 2016) with associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

similarity with those from Eastern and Sub-Saharan Africa suggests a common point of origin for the Eastern and Sub-Saharan African MCMV isolates. This is a piece of evidence that coat protein is highly conserved and an indication that MCMV virus has not undergone a lot of variation. The low variation could be attributed to the natural selection pressure which has been reported to influence the genetic variation of a virus (Roossinck and Schneider, 2006). The virus is still adapting to the new conditions and environment as a result of its newness in the area as it was first reported in September 2011 in Kenya (Wangai et al., 2012). The sequences were also similar with China isolate (GU594293), and Thailand isolates (AM490791). These findings corroborate with Adams et al. (2012) and Mahuku et al. (2015). They also reported a high degree of similarity of CP sequences of MCMV within Eastern Africa and some Asian MCMV isolates.

In the phylogenetic tree containing the SCMV sequences (Figure 3), two isolates KCO5 (accession #MH645623) and KCO24 (accession #MH645624) shared a high degree of identity. In contrast, one isolate, KCO59 (accession #MH645625), had high similarity with the Kenyan isolate (JX286708), which had been previously reported. These results indicate the presence of two groups of SCMV sequences in the region. It suggests they may be new variants of SCMV in the South-Rift region. These findings concur with Gao et al. (2011) and Perera et al. (2009), who found out that new strains of SCMV continue to be reported in many countries. Therefore, these results reveal that SCMV is more diverse than MCMV in the region. The diverse nature of SCMV could be attributed to the fact that SCMV had been reported in Kenya as early as the 1970s (Louie, 1980) and in Eastern Africa in the mid-1930s (Kulkarni, 1972) and therefore it may have undergone some variations with time. Also, the heavy maize losses as high as 50% reported by De Groote et al. (2016) in areas between Kisii and Kericho could be attributed to the new variants of SCMV in the region. Besides, the diverse nature of SCMV in the region could also be attributed to the extensive- scale cultivation of SCMV susceptible crops in Poaceae family (maize, sorghum, sugarcane, napier grass, pasture grasses). This has have been reported by Wu et al. (2012) as facilitating build-up of SCMV in fields and dissemination of the virus over large

areas in many countries worldwide.

Conclusion

This research has shown that SCMV is the main potyvirus in synergism with MCMV in causing MLN disease in Kenya. It has also demonstrated that MCMV isolates (accessions #MH645621 and #MH645621) from this study share a common origin with the MCMV isolates from Eastern and Sub-Saharan Africa. Further, the research has revealed that SCMV is more diverse than MCMV in the South-Rift region due to the existence of new variants of SCMV.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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