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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Use of simple sequence repeat (SSR) markers to establish genetic relationships among cassava cultivars released by different research groups in Ghanaian

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Cassava (*Manihot esculenta*) is an important staple crop widely cultivated in Ghana. The crop also has diverse industrial applications including starch, beer and alcohol productions. Knowledge about the state of the Ghanaian cassava genetic diversity and population structure is paramount in breeding programmes aimed at cultivar improvements or breeding of new cultivars for specific purposes. This study focused on the use of 36 simple sequence repeats (SSRs) to produce SSR allelic polymorphisms for estimation of inter- and intra-population genetic diversity among Ghanaian cassava cultivars from five Ghanaian released and local cassava populations consisting of 11 released and two local cultivars. The results show high diversity among the studied cultivars with an average of seven (7) alleles per locus. Polymorphic loci varied from 68.6 to 100% with an average of 88.58%. A strong genetic diversity was observed within populations (HS =0.552) and therefore suggesting a low rate of inter-population gene flow among the individuals constituting the populations. This high genetic variability among the cultivars provides valuable genetic resource to support any future breeding programmes aimed at establishing new cassava varieties for domestic and industrial purposes.

Key words: Cassava, DNA fingerprinting, genetic diversity, simple sequence repeat (SSR).

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the family Euphorbiaceae containing 28 wild spp. (Raghu et al., 2007), is one of the most important food crops of sub-Saharan Africa and grown throughout the tropics including

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Table 1. Five cassava populations involved in the study: "*CRI*", "*Professor Safo Kantanka*", "*Professor J.P Tettey*", "*SARI*" released materials and Local cultivars.

Cassava population	Breeder	Constituting cultivars
Population 1	Crops Research Released (CRI) materials	*Agbelifia*, *Essam Bankye*, *Afisiafi*, *Doku* Duade, *Bankye Hemaa*
Population 2	Professor Safo Kantaka released materials	*IFAD* and *Nkabom*
Population 3	Professor J.P Tettey released materials	*UCC* and *Bankye Botan*
Population 4	Savanna Agriculture Research Institute (SARI) released materials	*Filindiakoh* and *Nyerikobga*
Population 5	Local cultivars	*Akosua Tumtum* and *Debor*

Asia and Latin America. Cassava plays a famine prevention role wherever it is cultivated widely because it provides a stable base to the food production system (Jarvis et al., 2012; Romanoff and Lynam, 1992). In Ghana, cassava is produced in all but two regions in the northernmost part of the country and according to Nweke et al. (1999), in Collaborative Study of Cassava in Africa (COSCA), villages that did not experience the famine of 1983 in Ghana were those that cultivated cassava as the most important and dominant staple crop. Among the many cassava products in Ghana include fufu, gari, agbelima, agbelikaklo,yakeyake and kokonte (Wareing et al., 2001).

Cassava is assumed to have evolved from interspecific hybridization among its wild species. The crop is strongly an out crossing monoecious species but suffers from inbreeding depression, making it difficult to develop appropriate stocks for classical improvement of genetic methods (Fregene et al., 1997). The issue of inbreeding has led to the use of crossing blocks for inter mating superior individuals so that inbreeding could be minimized (Falahati-Anbaran et al., 2006).

Cassava is a diploid plant with 2n=36 chromosomes and a DNA content of 1.67 pg per cell nucleus (Prochnik et al., 2012; Awoleye et al., 1994). This is equivalent to 772 Mbp per haploid genome, and occupying the lower end of the genome size range for higher plants (Bennett et al., 1992). This relatively small genome size of cassava, favors development of saturated genetic map and molecular tag which may contribute to understanding of the inheritance of many important genetic traits despite the heterozygous nature of cassava (Fregene et al., 1997). Considerable amounts of genetic variations in cassava germplasm have been reported by many laboratories (Raghu et al., 2007; Acquah et al., 2011; Kawuki et al., 2013).

In recent years, there has been increasing interest in the use of DNA-based markers which is acclaimed to be more reliable marker for genetic diversity studies in contrast to the classical morphological markers. Morphological descriptors are highly subjective, environmentally influenced and of low polymorphism. However, due to the lack of molecular genetic expertise and high cost of establishing modern biotechnolical laboratories, many researchers continue to rely upon morphological descryptions for genetic diversity studies (Falahati-Anbaran et al., 2006). Simple Sequence Repeat (SSR) has been reliably used to quantify genetic diversity and examine popu-lation differentiation in a number of agricultural crops (Morgante et al., 1994; Maughan et al., 1995; Raghu et al., 2007). SSRs are co-dominant and tend to have multiple alleles per locus so that individuals can be identified as homozygotes or heterozygotes. An additional benefit of SSR marker is its ability to detect variations in allele frequency at many unlinked loci which are abundant in plants and the technique is easily adaptable to automation (Donini et al., 1998).

The objective of this study was to use SSR allelic polymorphisms produced by 36 SSRs to estimate inter- and intra-population genetic diversity among eleven (11) released and two (2) local Ghanaian cassava cultivars sampled from five cassava populations derived from local breeders.

MATERIALS AND METHODS

Plant materials

Five Ghanaian cassava populations involved in this study included 11 released and two local cultivars (Table 1). They were collected from farms of the CSIR-Crop Research Institute (CRI) at Fumesua in the Ashanti Region. Fresh stem cuttings 20-30 cm with 5-8 nodes were obtained from disease-free matured cassava plants. They were planted in loamy soil for a period of five weeks in plastic pots at the CSIR-Crops Research Institute screen house. The growing

Table 2. SSR primers and thermocycler programmes used in the study.

SSR Locus	Forward primer	Reverse primer	Thermocycler programme
SSRY 4	ATAGAGCAGAAGTGCAGGCG	CTAACGCACACGACTACGGA	MicroBC1
SSRY 9	ACAATTCATCATGAGTCATCAACT	CCGTTATTGTTCCTGGTCCT	MicroBC1
SSRY 12	AACTGTCAAACCATTCTACTTGC	GCCAGCAAGGTTTGCTACAT	MicroBC1
SSRY 19	TGTAAGGCATTCCAAGAATTATCA	TCTCCTGTGAAAAGTGCATGA	MicroBC1
SSRY 20	CATGGACTTCCTACAAATATGAAT	TGATGGAAAGTGGTTATGTCCTT	MicroBC1
SSRY 21	CCTGCCACAATATTGAAATGG	CAACAATTGGACTAAGCAGCA	MicroBC1
SSRY 34	TTCCAGACCTGTTCCACCAT	ATTGCAGGGATTATTGCTCG	MicroBC1
SSRY 38	GGCTGTTCGTGATCCTTATTAAC	GTAGTTGAGAAAACTTTGCATGAG	MicroBC1/ NewBC1
SSRY 51	AGGTTGGATGCTTGAAGGAA	GGATGCAGGAGTGCTCAACT	MicroBC1/Yucadiv (Yu)
SSRY 52	GCCAGCAAGGTTTGCTACAT	AACTGTCAAACCATTCTACTTGA	MicroBC1/Yu
SSRY 59	GCAATGCAGTGAACCATCTTT	CGTTTGTCCTTTCTGATGTTC	MicroBC1
SSRY 63	TCCAGAATCATCTACCTTGGCA	AAGACAATCATTTTGTGCTCCA	MicroBC1/Yu
SSRY 64	CGACAAGTCGTATATGTAGTATTCAG	GCAGAGGTGGCTAACGAGAC	MicroBC1/Yu
SSRY 69	AGATCTCAGTCGATACCCAAG	ACATCCGTTGCAGGCATTA	NewBC1(Ne)
SSRY 82	TGTGACAATTTTCAGATAGCTTCA	CACCATCGGCATTAAACTTG	MicroBC1/Yu
SSRY 100	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	NewBC1
SSRY 102	TTGGCTGCTTTCACTAATGC	TTGAACACGTTGAACAACCA	NewBC1
SSRY 103	TGAGAAGGAAACTGCTTGCAC	CAGCAAGACCATCACCAGTTT	NewBC1
SSRY 105	CAAACATCTGCACTTTTGGC	TCGAGTGGCTTCTGGTCTTC	NewBC1
SSRY 106	GGAAACTGCTTGCACCAAAGA	CAGGCAAGACCATCACCAGTTT	NewBC1
SSRY 108	ACCCTATGATGTCCAAAGGC	CATGCCACATAGTTCGTGCT	MiccroBC1/Yu
SSRY 110	TTGAGTGGTGAATGCGAAAG	AGTGCCACCTTGAAAGAGCA	NewBC1
SSRY 135	CCAGAAACTGAAATGCATCG	AACATGTGCGACAGTGATTG	Yucdiv
SSRY 147	GTACATCACCACCAACGGGC	AGAGCGGTGGGCGAAGAGC	Yucadiv
SSRY 148	GGCTTCATCATGGAAAAACC	CAATGCTTTACGGAAGAGCC	Yucadiv
SSRY 151	AGTGGAAATAAGCCATGTGATG	CCCATAATTGATGCCAGGTT	NewBC1
SSRY 155	CGTTGATAAAGTGGAAAGAGCA	ACTCCACTCCCGATGCTCGC	Yucadiv
SSRY 161	AAGGAACACCTCTCCTAGAATCA	CCAGCTGTATGTTGAGTGAGC	Yucadiv
SSRY 164	TCAAACAAGAATTAGCAGAACTGG	TGAGATTTCGTAATATTCATTTCACTT	NewBC1
SSRY 169	ACAGCTTAAAAACTGCAGCC	AACGTAGGCCCTAACTAACCC	Yucadiv
SSRY 171	ACTGTGCCAAAATAGCCAAATAGT	TCATGAGTGTGGGATGTTTTTATG	NewBC1
SSRY 177	ACCACAAACATAGGCACGAG	CACCCAATTCACCAATTACCA	Yucadiv
SSRY 179	CAGGCTCAGGTGAAGTAAAGG	GCGAAAGTAAGTCTACAACTTTTCTAA	MicroBC1
SSRY 180	CCTTGGCAGAGATGAATTAGAG	GGGGCATTCTACATGATCAATAA	MicroBC1
SSRY 181	GGTAGATCTGGATGGAGGAGG	CAATCGAAACCGACGATACA	Yucadiv
SSRY 182	GGAATTCTTTGCTTATGATGCC	TTCCTTTACAATTCTGGACGC	Yucadiv

conditions used were 30ºC day temperature, 24ºC night temperature, 12 h day light, and 55% relative humidity. Plants were watered daily and the experiment repeated in the following year with similar growth conditions.

DNA isolation and polymerase chain reaction

Genomic DNA was isolated from cassava leaves according to Egnin et al. (1998) isolation protocol adopted by the Council for Scientific and Industrial Research- Crops Research Institute (CSIR-CRI) molecular laboratory with some modifications. DNA quality was determined on 0.8% agarose gel stained with ethidium bromide. 10 µl PCR reaction mixtures [1.0 µl of buffer (10X), 0.9 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTPs (10 mM), 0.25 µl of both forward and reverse primer (10 µM), 0.125 µl Taq polymerase (5 U), 1.0 µl of genomic DNA template (10 ng/ µl) all together with 6.075 µl of nuclease-free PCR water] were prepared. Amplification was performed for 30 cycles in MyCycler thermal cycler (Bio-Rad Laboratories Inc.) with heated lid to reduce evaporation. The 36 SSR markers used were in three cycling programmes namely: Yucadiv, MicroBC1 and NewBC1 (Table 2).

The following were the cycling profiles: Yucadiv [95°C for 2 min (initial denaturation), 30 cycles of the following steps: 94°C for 30 s

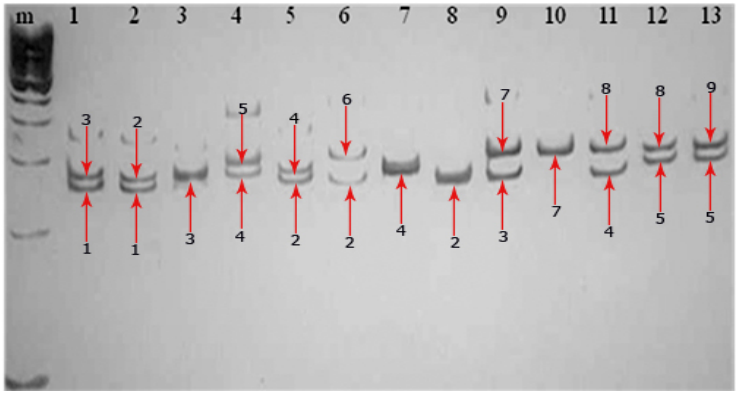

Figure 1. 6% Polyacrylamide gel electrogram showing silver stained PCR amplified allelic fragments of locus SSRY 59 for 11 released and two local Ghanaian cassava cultivars. M, 100 bp marker; 1, *UCC*; 2, *IFAD*; 3, *Agbelefia*; 4, *Nyerikobga*; 5, *Nkabom*; 6, *Esaam Bankye*; 7, *Akosua Tumtum*; 8, *Debor*; 9, *Filindiakoh*; 10, *Afisiafi*; 11, *Doku Duade*; 12, *Bankye Hemaa*;13, *Bankye Botan*.

(denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension) then 72 C for 5 min (final extension) and storage at 4°C]; MicroBC1 [94°C for 2 min (initial denaturation), 30 cycles of the following steps: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension) then 72°C for 5 min (final extension) and storage at 4°C]; NewBC1 [95°C for 2 min (initial denaturation), 30 cycles of the following steps: 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 1 min (extension) then 72°C for 5 min (final extension), 25 cycles of the following steps; 94°C for 30 s, 65°C for 30 s (with annealing decreasing subsequently by 1°C/ Cycle), 72°C for 1 min then 94°C for 30 s (final denaturation), 55°C for 30 s (annealing), 72°C for 1 min (final extension) and storage at 4°C].

Gel electrophoresis and band scoring

After the amplification reactions, 8 µl of the amplified DNA fragments were separated on 6% PAGE gel [12 % acrylamide solution (19:1), TBE (12X) 0.4% APS, TEMED and filtered autoclaved distilled water (FADW)] at 200 V for 35 min in TBE (1X) using a mini-protean 3 cell electrophoretic apparatus. A 100 bp DNA marker (0.05 µg/µl, 25 µg) (Invitrogen) was used as a standard and the DNA fragments were visualized by silver nitrate staining (Figure 1).

The SSR amplified bands were scored as diploid (each individual cultivars score at a locus consisted of two digits) and codominant (each separate allele at a locus was given a score that relates to its size) by visual inspection and “climbing ladder” approach for both F-Statistics (FSTAT) and TOOLS FOR Population Genetic Analysis (TFPGA). For example, in Figure 1 Lane 1, the second band (1) corresponds to an allele seen on a chromosome while the first band (3) corresponds to the alternative allele, with a higher bp than the second allele, located at that same locus. For TFPGA analysis, cultivar 8 and 10 (*Debor* and *Afisiafi*) for example were scored as 0202 and 0707 respectively meaning the cultivars were identified at locus SSRY 59 as homozygous for alleles 2 and 7 respectively (Figure 1). On the other hand, cultivar 6 (*Essam bankye*) was represented as heterozygous for alleles 2 and 6 and this was scored as 0206. However, in FSTAT analysis the scoring 0202 and 0206 would be recorded as 22 and 26 respectively.

Statistical analysis and genetic diversity determination

The bands score data were analyzed for descriptive statistics parameters [number of alleles per locus, allelic richness, allelic frequency, percent polymorphic loci, Hardy-Weinberg equilibrium, gene diversity per locus per population and Nei's (1978) estimation of heterozygosities], F-statistics and cluster analysis, and Nei's (1978) genetic distance. With the exception of Hardy-Weinberg equilibrium, cluster analysis, Nei's (1978) genetic distance and F-statistics which were analyzed using TFPGA version 1.3 (Miller, 1997) all other analysis were carried out with FSTAT version 9.3.2 (Goudet, 1995).

RESULTS

DNA isolation, PCR and gel electrophoresis

High quality genomic DNA assessed on 0.7% agarose gel were successfully isolated from all the 13 cassava cultivars. A total of 35 out of the 36 primers, representing 97.2%, produced clear and scorable bands in electrophoretic gels (Figure 1). Amplification at locus SSRY 177 failed in all the triplicate reactions. *Afisiafi* generated no products at locus SSRY 38, 106 and 164. Similarly, at locus SSRY 179 and 180 no PCR products were observed for *Agbelifia* cultivar. Again there were no successful PCR products in *Nkabom* cultivar at locus SSRY 52. For the 13 cultivars analyzed, all the 35 microsatellite loci were found to be polymorphic.

Genetic diversity determination

The number of alleles observed per locus, at all the 35 SSR loci analyzed, varied from three (3) to eleven (11) and averaging seven (7) alleles per locus. The number of

Table 3. The number of alleles and percentage polymorphic loci recorded among the cassava populations under study

Cassava Population	Breeders	Total number of allele per population	Percentage of polymorphic loci per population
Population 1	Crops Research Released (CRI) materials	163	100
Population 2	Professor Safo Kantaka released materials	86	80.0
Population 3	Professor J.P Tettey released materials	99	100
Population 4	Savanna Agriculture Research Institute (SARI) released materials	93	94.3
Population 5	Local cultivars	66	68.6
Mean		101.4	88.58

Table 4. Mean values of Nei's (1978) estimation of heterozygosity.

Ho	He	Ht	Dst	Gst
0.497	0.769	0.814	0.044	0.054

Ho, Observed heterozygosity; He, expected heterozygosity; Ht, total heterozygosity; Dst, interpopulation gene diversity; Gst, coefficient of genetic differentiation.

Table 5. Unbiased heterozygosity of individual populations.

Populations	Heterozygosity
Pop. 1	0.795
Pop. 2	0.643
Pop. 3	0.800
Pop. 4	0.729
Pop. 5	0.481
Mean	0.699

Population 1, *CRI* released materials; population 2, *SARI* released materials; population 3, *Prof. J.P. Tettey* released materials; population 4, *Prof. Safo Kantanka* released materials; population 5, local cultivars.

alleles per polymorphic population ranged from 66 to 163 with an average of 101.4 whereas the percentage of polymorphic loci population varied from 68.6 to 100% and averaging 88.58% (Table 3).

Nei's diversity indices estimation showed a low value of 0.497 for the observed heterozygosity (HO) whereas the expected heterozygosity (He) on the other hand recorded a high value of 0.769 (Table 4). However, a high total heterozygosity (Ht) of 0.814 was observed in all the 13 cassava cultivars. The results also established inter-population gene diversity (Dst) of 0.044 and coefficient of genetic differentiation (Gst) value of 0.054. A heterozygote deficit of 0.37 was observed. Hardy-Weinberg equilibrium also recorded 0.273. The unbiased heterozygosities of the individual populations recorded values ranging from 0.4810 to 0.800 (Table 5). The local cultivars recorded the lowest heterozygosity while *Prof. Tettey* released materials recorded the highest (Table 5). Nei (1978) genetic distance ranged from 0.1440 to 1.0057 (Table 6) whereas the genetic diversity revealed a high genetic variability range of 0.500 to 0.94.3 (Table 7).

Table 8 contains outputs of the Wright's F-statistical analysis. The results established a high correlation of genes within individuals (Fit), heterozygote deficit among individuals within each population (Fis) and a high occurrence of cross pollination (C) at 0.404, 0.372 and 0.663 respectively. The correlation of genes of different individuals in the same population (Fst) and the fixation index coefficient (F) obtained were however low at 0.052 and 0.373 respectively. The analysis also showed low occurrence (1.028) of inter-population gene flow (Nm).

DISCUSSION

The study has clearly shown high genetic variability among the five Ghanaian cassava populations assessed by 36 SSR with an average of seven (7) alleles per locus. The observed genetic variability among the cultivars contrasts sharply with an earlier work conducted by Okai et al. (2003) in which they claimed to have observed low genetic variability among 320 cassava landraces from Ghana. The high variability among the cassava populations studied attest to the use of genetically diversified progenitors for the breeding and establishment of the cassava cultivars bred for their unique nutritional and industrial benefits (Manu-Aduening et al., 2005).

Table 6. Nei (1978) unbiased distance.

Population	1	2	3	4	5
1	*****				
2	0.5748	*****			
3	0.2732	0.5541	*****		
4	0.1440	0.3408	0.4053	*****	
5	0.6477	0.7757	1.0057	0.5197	*****

Population 1, *CRI* released materials; population 2, *SARI* released materials; population 3, *Prof. J.P. Tettey* released materials; population 4, *Prof. Safo Kantanka* released materials; population 5, local cultivars.

Table 7. Gene diversity per locus and population.

Pop 1	Pop 2	Pop 3	Pop 4	Pop 5
0.835	0.676	0.943	0.850	0.500

Pop 1, *CRI* released materials; Pop 2, *SARI* released materials; Pop 3, *Prof. J.P. Tettey* released materials; Pop 4, *Prof. Safo Kantanka* released materials; Pop 5, local cultivars.

Table 8. Output of the Wright's F statistics.

Fit	Fst	Fis	F	Nm	C
0.404	0.052	0.372	0.373	1.028	0.663

Means were obtained for F-statistics parameters. The parameters analyzed were correlation of genes within individuals overall population (Fit), correlation of genes of different individuals in the same population (Fst), heterozygote deficit observed in individuals within each population (Fis), fixation index coefficient (F), estimation of gene flow (Nm) and frequency of cross pollination (C).

The high number of alleles per locus and the corresponding high number of alleles per population averaging over 100, contributed to the high polymorphic loci observed (88.58%). A collection of cassava cultivars with such high values is expected to have wide genetic distances between their constituting cultivars. Nei (1978) genetic distance of 0.1440 to 1.0057 was however expected. This wide genetic distance implies that the cassava varieties from the five populations used in the study possess very diverse genetic backgrounds (da Costa et al., 2013). Extensive diversity in their progenitor is most likely source of genetic variations in the collection considering the young age of the varieties. The large genetic distances observed among the cultivars support effective breeding of new varieties from the existing ones. These cultivars still remain potential progenitors for breeding of new cassava cultivars for various purposes.

It is clear in this study that the probability of any two randomly selected alleles from any given population could be different that is (He) 0.769 or 76.9%. This value was observed to be higher than what was obtained in other related works on cassava (Lokko et al., 2006; Fregene et al., 2003). The value is far higher than what have been observed in outcrossing species of dicots (0.159) and all other plant species (0.205) (Hamrick and Godt, 1997). These, notwithstanding Nei's estimation of observed heterozygosity, recorded a smaller value of 49.7% resulting in a heterozygote deficit of 27.2% among the cultivars. The F statistics analysis, Fis 0.372, Fit 0.404 and F at 0.373 confirmed the observed deficit (Gehring and Lindhart, 1992; Dolan et al., 1999). However, the deviation from Hardy-Weinberg equilibrium (0.273) indicating excess of homozygosity and existence of a non-random mating among the cultivars explained the low observed heterozygosity which resulted in the 27.2 % loss of genetic diversity.

These observations could be influenced by the extensive vegetative cultivation of cassava and the rare incidence of genetic self-incompatibility within the crop. Although inflorescences are metandrous, seed dispersal in cassava via explosive mechanism does not promote long distance gene flow (Olsen and Schaal, 2001). These reasons could favor inbreeding and thus affecting cassava breeding due to narrowing of the cassava genetic base (Okai et al., 2003). A narrow genetic base restricts the progress of cultivar improvement by compounding the difficulty involved in choosing parental materials for breeding. That is, narrow genetic base is undesirable for breeding because a certain level of parental divergence is needed to create productive hybrids (Miranda et al., 2008).

Though, Nei (1978) genetic diversity revealed high genetic variability (0.500 to 0.94.3) among the populations studied in this work, a higher intra-population diversity was recorded (HS = 0.552) as compared to inter-population diversity (low Gst 0.054, FST 0.052 and Dst 0.044). This suggests that the populations assessed in our study have a low rate of inter-population gene flow, that is Nm 1.028, which could possibly lead to a low genetic overlap as a result of minimal gene flow through seed and pollen. Faraldo et al. (2000), Mühlen et al. (2000),

Asante and Offei (2003), Peroni et al. (2007) and Lokko et al. (2006) all observed similar results as they reported high intra-genetic variability in Ghanaian cassava varieties. A similar pattern was observed with sweet potato land-races from the Vale do Ribeira (Veasey et al., 2008). On the contrary, the high genetic variation within the Ghanaian released cassava populations indicates the usefulness of each of the four local populations as a valuable genetic resource for the selection of superior genotypes, as seen in the case of 'CRI released materials, Prof. J.P. Tettey released materials and Prof. Safo Kantanka released materials' showing 83.5, 85.0 and 94.3% genetic diversity respectively.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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