Assessment of Genetic Variation Among Some Introduced Tomato (*Solanum lycopersicum L.*) Varieties Using SSR markers

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Abstract
Simple sequence repeats (SSR) is one of the most appropriate markers for variety identification as it has immense discrimination influence for varieties with restricted genetic variation. Total genomic DNA was extracted from young freshly leaves of five tomato varieties. Genetic characterization of introduced tomato varieties were investigated using 15 SSR markers. Out of the 15 markers, 14 gave polymorphic bands and therefore were considered for further analysis. The number of alleles ranged from 2.00 (SSR2), (SSR3) to 6.00 (SSR9), (SSR11) alleles per locus with a mean value of 4.07 alleles per locus. The average Polymorphic Information Content (PIC) value was 0.59 ranging from 0.31(SSR2) to 0.77 (SSR9). The most polymorphic primers were SSR9, SSR11, and SSR4 based on PIC values. The Agglomerative hierarchical clustering grouped the tomato varieties into two (A & B). At a coefficient of 0.88, cluster B had three sub-clusters BI (Heinz), BII (Shasta) and BIII (OP-B155 and CRI-P00). At a coefficient of 0.90 varieties OP-B155 and CRI-P00 were identified as the most genetically related varieties. The genetic distance information from this study might be useful for further implementation of breeding strategies.

Key words: genetic variability, Solanum lycopersicum, simple sequence repeat, maker

Résumé
Répétitions de séquences simples (SSR) est l'un des marqueurs les plus appropriés pour l'identification des variétés comme il a une immense influence de la discrimination pour les variétés avec la variation génétique restreinte. L'ADN génomique total a été extrait de jeunes feuilles fraîchement des cinq variétés de tomates. La caractérisation génétique des variétés de tomates introduites ont été étudiées à l'aide de 15 marqueurs SSR. Sur les 15 marqueurs, 14 ont donné des bandes polymorphes et donc ont été considérés pour une analyse ultérieure. Le nombre d'allèles variait de 2,00 (SSR2), (SSR3) à 6,00 (SSR9), (SSR11) allèles par locus avec une valeur moyenne de 4,07 allèles par locus. La valeur moyenne de contenu d'information polymorphique (PIC) était de 0,59 allant de 0,31 (SSR2) à 0,77 (SSR9). Les plupart des amorces polymorphes étaient SSR9, SSR11 et SSR4 fondée sur des valeurs de PIC. La classification ascendante hiérarchique a regroupé les variétés de tomates en deux (A & B). A un coefficient de 0,88, le groupe B avait trois sous-groupes BI (Heinz), BII (Shasta) et

BIII (OP-B155 et CRI-P00). A un coefficient de 0,90 variétés OP-B155 et CRI-P00 ont été identifiés comme les variétés les plus génétiquement liés. L'information génétique à distance de cette étude pourrait être utile pour poursuivre l'application de stratégies de sélection.

Mots clés: variabilité génétique, Solanum lycopersicum, répétition de séquence simple, maqueur

Introduction

Tomato is one of the main vegetable crops all over the world, contributing both vitamin A and vitamin C to the human diet and providing high economic value to producers and breeding industries in many countries (FAO, 2003). In addition to its worldwide agricultural and economic importance as a crop, tomato is a pre-eminent model system for genetic studies in plants. In the course of domestication, as a result of intensive selection, the genetic diversity of cultivated tomato varieties has become narrower than in other crops (Osei *et al.,* 2009). The selection of cultivated tomato *(Solanum lycopersicum L.)* has followed two main directions; improvement of disease resistance and fruit quality, assisted by introgression of genes from the wild Lycopersicon species (Santiago and Caballero, 1995). Nonetheless, tomato is known as a crop characterized by narrow genetic diversity. Less than 10% of the total genetic diversity in the Lycopersicon gene pool is found in *S. lycopersicum.* This makes the identification of the best parental genotypes for crosses and the development of effective breeding strategies difficult (Tanksley and Miller, 1990). That is why the study of genetic diversity in elite tomato germplasm is one of the most important issues with an enormous impact on the effective management of genetic resources. The identification of variability among accessions is vital to the maintenance, utilization and acquisition of germplasm resources (Mwirigi, 2009). The genetic diversity analysis is a descriptive technique which reveals the pattern of character variation among individual accessions (Mwirigi, 2009). On the other hand agglomerative hierarchical clustering decreases the number of individual variable units by classifying such variation into groups which are translated into a dendrogram using the coefficient of similarity (IPGRI and Cornell University, 2003). The allele frequency analysis calculates two common measure of variation for each locus namely, expected heterozygosity and polymorphic information content (Otoo *et al.,* 2009). Currently, most of the molecular markers used for assessment of genetic diversity, intervarietal differences and varietal identification in tomato, are PCR-based. These include RAPD (random amplified polymorphic DNA), SSR (simple sequence repeat, or microsatellite), ISSR (inter-simple sequence repeat), AFLP (amplified fragment length polymorphism), SCAR (sequence-characterized amplified region), CAPS (cleaved amplified polymorphic sequence), SNP and In Del markers. Among these markers, SSRs stand out as highly informative (Bredemeijer *et al.,* 1998; Cooke *et al.,* 2003)] and, unlike SNPs, do not require knowledge of the amplified sequences, further enzymatic manipulations (Schlotterer, 2004) or high-throughput technologies. The main advantage of SSRs is their co-dominant type of inheritance. With co-dominant markers, heterozygous individuals can be easily distinguished from homozygous, allowing the determination of allele configuration of each genotype and allele frequencies at different loci (Parmar *et al.,* 2013). During recent decades, Simple sequence repeats (SSR) also known as microsatellites have become the most popular source of genetic markers owing to their high reproducibility,

multi-allelic nature, abundance, and wide genome coverage. SSR markers have been successfully adopted to analyze genetic diversity in a variety of different plant species (McCouch *et al.,* 1997; He *et al.,* 2003; Frary *et al.,* 2005; Sarıkamış *et al.,* 2006, 2009, 2010). It was long assumed that SSRs were primarily associated with non-coding DNA, but it is now clear that they are also abundant in the single- and low- copy fraction of the genome. SSRs, however, require high-resolution fluorescent-based capillary systems to fully expand their informative potential. Cultivated tomato (*S. lycopersicum* L.) is known to be highly monomorphic at the molecular level although it is phenotypically very diverse (Luikart *et al.,* 1998). Genetic diversity in tomato is useful in the selection. In this study the genetic diversity of five tomato varieties from the USA including a local line from Ghana was assessed using fluorescence based SSR genotyping in order to reveal and explore the genetic variation available in them.

Materials and methods

Plant materials

The plant material for the study consisted of four tomato varieties obtained from the USA and one local material from Ghana. The seeds were kindly provided by the CSIR - Crops Research Institute, Kumasi, Ghana (Table 1).

SSR primers

Fifteen (15) microsatellite primer pairs obtained from Metabion International laboratory, Germany were used for the genotyping. Primer names, sequences and corresponding number of bases are listed in Table 2.

Table 1: Names of tomato varieties and their source

Variety	*Source*	*Type*
Shasta	USA	Hybrid
Heinz	USA	Hybrid
OP-B155	USA	Open-pollinated
OP-B149	USA	Open-pollinated
CRI-P00	Ghana	Breeding line

Genomic DNA extraction

Genomic DNA was extracted from young freshly harvested leaves of the tomato varieties from the field using a modified DNA isolation method described by Takrama, 2000. Tender tomato leaves of 20 mg were weighed into 2 ml eppendorf tube, ground to fine powder in liquid nitrogen using Teflon pestle. To each tube, 800 µ? buffer containing 2 g CTAB, 2 g PVP, 28 ml NaCl, 4 ml EDTA (pH 8.0), 10 ml Tris HCl (pH 8.0), and 0.1 ml 2 mercaptoethanol was added and shaken several times until a homogenous mixture was obtained so as to lyse nuclear membranes. The protein contaminants from the cell lysate were removed by adding equal volume (800 µl of chloroform isomyl alcohol as used by Takrama, 2000 and mixed gently by inversion of the tube. DNA containing phase transferred into fresh tubes without disturbing the tube. An equal volume of ice cold isopropanol, which had been stored at -20°C, were added to each tube with the DNA containing supernatant and the tube gently inverted thrice to precipitate the DNA. The samples were incubated at -20°C for 8 hours. The samples were centrifuged again at 14000 rpm for 5 minutes, the supernatant was discarded and the DNA pellet washed with 500 µl of 80% ethanol. The samples were centrifuged at 6000 rpm for 4 minutes. DNA pellets were air-dried at room temperature for 10 minutes to remove the remaining ethanol droplets from the tube and redissolved in 50 µl of 1X TE (Tris and EDTA) buffer. RNase A of 1µl (6mg/ml) was added to the redissolved DNA samples and incubated at 37°C for 10 minutes to remove any RNA remaining. The DNA was purified with 400 µl of 6M bromophenol blue, incubated on ice for 30 minutes and then centrifuged at 14000 rpm for 5 minutes. The supernatant was transferred

Table 2: Set of tomato microsatellite markers used in DNA fingerprinting

S/No	*Marker Name*	*Primer sequence (5' - 3')*	*No. of Bases*
1	TGS0001F	GCGACCCTCTATTGAACTTGAAGAC (F)	25
		ACAAATCAAAGGAACAATTTCAA (R)	23
2	TGS0002F	GCAAACGTGTTCGAGTTCGTG (F)	21
		CCACACAATAAAGACAGAAAAATG (R)	24
3	TGS0003F	ATGCATGCGTGTGTGTTGTA (F)	20
		GTGTGTGTGTGTGTGTGTGTGT (R)	22
4	TGS0004F	GCAATTTATTTTCATTTGTTATACCGGA (F)	28
		ACCGAGACTCCTGGCTCATA (R)	20
5	TGS0005F	GACAAAAATTTTCCACACGGC (F)	21
		TCTCTTATAATTTTGTTGAGTCTCTGA (R)	27
6	TGS0006F	GTCGCATAAATATGGACAACGA (F)	22
		TTTTTAAAATACCATTCCAGAAAAA (R)	25
7	TGS0007F	GTGGATTCACTTACCGTTACAAGTT (F)	25
		CATTCGTGGCATGAGATCAA (R)	20
8	TGS0008F	GCGGTGTGAAATACAACAAGACG (F)	23
		CTCGACAAGCTAATTTCTGGG (R)	21
9	TGS0009F	GCGAAGCAAAAGAAAATTGGG (F)	21
		CACCACGAAGGCTGTTGTTA (R)	20
10	TGS00010F	TTGAAAAGCTGAAAAGTCAATCA (F)	23
		GAGAGGTGCCACATCACCTT (R)	20
11	TGS00012F	GTCCCTACCCCACAAATTGAA (F)	21
		AGGTACAACTCACCTCCCCC (R)	20
12	TGS00013F	GGTGGACATATGAGAAGACCTTG (F)	23
		TCATTTTCCAATGGTGTCAAA (R)	21
13	TGS00014F	GTGAAGACGAAAAACAAGACGA (F)	22
		CCTTCCCCTTTTGTCTCTCC (R)	20
14	TGS00020F	TCTTTCAACTTCTCAACTTTGGC (F)	23
		GCCGACTTCAAAAACTGCTC (R)	20
15	TGS00023F	GTCCAAATTAAAAACTAACCGCA (F)	23
		TTTCCAAAATGACCTAGCGG (R)	20

NB: F: Forward primer, R: reverse primer

into a clean 1.5 ml eppendorf tube and 14000 rpm for 15 minutes. The DNA pellets were washed with 400 µl of 80% ethanol, air-dried at room temperature and redissolved in 50 µl 1X TE (Tris-ethylenediaminetetracetic acid) buffer and centrifuged at high speed for 30 seconds to remove all insolubles.

Microsatellite genotyping

The genomic DNA of the samples were fingerprinted using SSR markers in a 10 µl reaction volume of master mix containing, 1.5 mM of 5X Buffer A with MgCl2, 10 mM of

dNTPs (Deoxynucleotide Triphosphates), 0.3 µM each of forward and reverse primer and 0.08 µl Taq polymerase. Water was added to make the final volume. Reactions were conducted at an initial denaturation at 95^{0}C for 5 min; denaturation at 95^{0}C for 30 sec; annealing 55^{0}C for 30 sec; extension at 72^{0}C for 30 sec; final extension at 72^{0}C for 5 sec a touchdown procedure for 35 cycles and then held at 4^{0}C. Reactions were carried out in Techne prime thermal cycler (Labnet International Inc., California, USA) and GeneAmp PCR System 9700 (Applied Biosystems, USA) of 96-well plates with heated lid to reduce evaporation. The amplified products were stored at -20^{0}C until required to run gels. The PCR products were separated using horizontal Agarose gel electrophoresis (AGE). A 100bp DNA marker (gene rule) was used as a reference to estimate the size of specific DNA bands in the PCR amplified products. After the AGE, the DNA fragments were visualized in the gels by 0.1% ethidium bromide and photo-documented with a digital camera under UV light.

Band scoring and cluster analysis

Banding patterns were then visualized and compared between individuals. All the genotypes (PCR products) were scored for presence (1) and absence (0) of the SSR bands. For each marker, alleles for the data set were scored according to size of base pairs of the 100 bp ladder DNA marker. This procedure was conducted for each marker until all alleles were scored with the smallest and largest sized alleles representing the start of the first scoring and the end of the last scoring, respectively. The data recorded was subjected to NTSYSpc 2.20 software (Jaccard's dis/similarity coefficient) to generate structure dendrogram. Power Markers computed programme, version 3.25 was also used in conducting allelic frequency analysis Liu and Muse (2005) used.

Results

Characteristics of SSR markers

In all, 15 microsatellite markers were used to test the genetic diversity of five tomato varieties. Out of the 15 primers, 14 primers gave polymorphic bands. The number of alleles ranged from 2.00 (SSR2), (SSR3) to 6.00 (SSR9, SSR11) alleles per locus with a mean value of 4.07 alleles per locus. The Polymorphic Information Content (PIC) values also ranged from 0.31 (SSR2) to 0.77 (SSR9) with an average of 0.59. The most polymorphic primers were SSR4, SSR9 and SSR11 based on PIC values. The allele frequency of the primers indicates that, SSR5 was not polymorphic and was therefore dropped. The rest were all polymorphic in character. Gene diversity was high ranging from 0.38 (SSR2) to 0.80 (SSR9) with a mean of 0.61. However, SSR8, SSR10 and SSR13 had the same Gene diversity of 0.66 with a mean value of 0.61 for all (Table 3).

The agglomerative hierarchical clustering dendrogram illustrates the relationship among the accessions (Fig. 1). Cutting the line at a similarity coefficient of 0.85, two main groups were obtained. Cluster A had only one variety (OP-B149) while cluster B had four varieties (OP-B155, CRI-P00, Shasta and Heinz). At a coefficient of 0.88, cluster B had three sub-clusters B? (Heinz), B?? (Shasta) and B??? (OP-B155 and CRI-P00). At a coefficient of 0.90 varieties OP-B155 and CRI-P00 were identified as the most genetically related varieties (very identical).

Discussion

The aim of this study was to characterize tomato genotypes using SSR markers. Even though, some SSR markers have been developed, their availability is limited. Recently, SGN has developed 609 SSR markers and assayed on *Solanum lycopersicum* and *Lycopersicon pennellii* (Frary *et al.,* 2005). Consequently, 15 SSR markers used for genetic characterization

Table 3: Allele frequency, Number of Allele, Gene Diversity, Heterozygosity and Polymorphic Information Content (PIC) values from SSR data

Marker	*Allele Frequency*	*Allele No.*	*Gene Diversity*	*Heterozygosity*	*PIC*
SSR1	0.7500	3.0000	0.4063	0.5000	0.3706
SSR2	0.7500	2.0000	0.3750	0.5000	0.3047
SSR3	0.2500	2.0000	0.5000	1.0000	0.3750
SSR4	0.5000	4.0000	0.7500	1.0000	0.7031
SSR6	0.2500	4.0000	0.6563	0.5000	0.6050
SSR7	0.6000	3.0000	0.5400	0.8000	0.4662
SSR8	0.4000	4.0000	0.6600	1.0000	0.5958
SSR9	0.3000	6.0000	0.8000	1.0000	0.7716
SSR10	0.4000	4.0000	0.6600	1.0000	0.5958
SSR11	0.3000	6.0000	0.7800	0.8000	0.7482
SSR12	0.6000	4.0000	0.5800	0.6000	0.5350
SSR13	0.5000	4.0000	0.6600	0.4000	0.6102
SSR14	0.5000	3.0000	0.6200	0.6000	0.547
SSR15	0.6000	5.0000	0.6000	0.6000	0.5700
Mean	0.4607	4.0714	0.6134	0.7357	0.5942

among the five tomato genotypes revealed some distinction and similarity level among them. The similarity level was due to the narrow genetic diversity, as has been previously stated by Suliman- Pollatschek *et al.* (2002) and He *et al.* (2003). The genetic diversity of the genotypes studied was evaluated by using 14 polymorphic SSR primers. In essence, the allele frequency analysis calculates two common measure of variation for each locus namely, expected heterozygosity and polymorphic information content (PIC). The expected heterozygosity measure is helpful in establishing the in formativeness of a locus. Loci with expected heterozygosity of 0.5 or less are not very useful for large-scale parentage analysis (Otoo *et al.,* 2009). The results of observed heterozygosity of the loci was greater than 0.5, in all the markers used for the study, expect in SSR13 (0.40) signifying that a good parentage analysis can be obtained from the molecular analysis. This observation also illustrates that, at a single locus, any two alleles, chosen at random from the population are different from each other (IPGRI and Cornell University, 2003). Additionally, the high observed heterozygosity values in this study confirm the heterozygote nature of most of the accessions studied (Obidiegwu *et al.,* 2009). Nevertheless, the average heterozygosity over all loci therefore estimates the extent of genetic variability in the population. The mean heterozygosity value of 0.74 reveals that there was some degree of genetic variation among the population (IPGRI and Cornell University, 2003).

Moreover, the efficiency of each primer was estimated by the number of alleles and discriminating power was calculated by the assessment of the polymorphic information content. PIC is regarded as one of the impor-

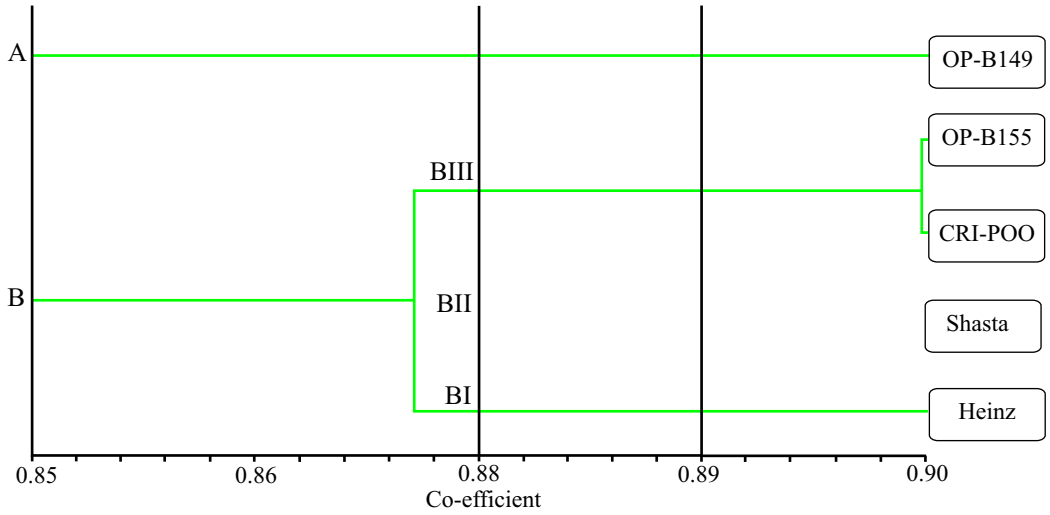

Figure 1: Dendrogram showing relationship among five tomato varieties.

tant features of molecular markers and can be used to evaluate the differentiation ability of the markers within the population (Junjian *et al.,* 2002). PIC is a measure of informativeness related to expected heterozygosity and is calculated from allele frequencies (Norman *et al.,* 2012). Such measurement is useful in linkage mapping studies. The results from the allelic frequency analysis generally implied that the loci revealed high polymorphism verified by elevated PIC values (0.31-0.77) (Table 2). Thus, the SSR markers used were efficient in discriminating the species. The amount of PIC is a function of detected alleles and the distribution of their frequency (Moghaddam, and Alikhani, 2009). Thus markers with more alleles and low allele frequency had larger PIC as found in SSR9 (6 alleles and the highest PIC of 0.77 followed by SSR11 (6 alleles and the PIC of 0.74) respectively indicating a better distinction of the accessions. These results confirmed the utility of PIC as a measure of the capacity of a marker to discriminate among closely related individuals as pointed out by Prevost and Wilkinson, (1999) and Escandon *et al.* (2007). PIC values demonstrated that the SSRs used in the study were highly informative. The mean PIC value recorded in this study however differs from results obtained from previous studies by Bredemeijer *et al.* (2002) who reported that the number of alleles per locus ranged from 2 to 8 with an average of 4.7 alleles per locus in 521 tomato varieties. He *et al.* (2003) found 2 to 6 alleles for each locus after testing 65 SSR loci on 19 tomato accessions. Similarly, Garcia-Martinez *et al.* (2006) reported that number of SSR alleles detected in 48 tomato accessions ranged from 2 to 10 alleles for the 19 SSR markers. In addition, PIC mean values (0.59) for this study was greater than those recorded He *et al.,* (2003) (0.37), Bredemeijer *et al.,* (2002) (0.40), Frary *et al.,* (2005) (0.39) and less Garcia-Martinez *et al.,* (2006) (0.78). The result of this study therefore showed that all the primers were highly informative and can be used for genetic diversity studies and the study of phylogenetic relationship. The markers SSR1, SSR2, SSR7, SSR12 and SSR15 (Table 2) had the highest frequencies of 0.75, for SSR1 and SSR2, 0.60, for SSR7, SSR12 and SSR15 respectively, while SSR4, SSR9, SSR11 and SSR8, SSR10 (Table 2) had the lowest frequencies of the predominant allele (0.25, for SSR4, 0.30, for SSR9 and

SSR11 and 0.40 for SSR8 and SSR10 respectively). Low frequency of the predominant allele reveals the suitable allelic distribution among the accessions (Priolli *et al.,* 2002). SSR markers with the higher number of alleles per locus showed the lowest frequency of the predominant allele, thus, markers with lower frequency of the predominant allele have more differentiation ability than other markers. Large number of alleles per locus observed (4.07, on average, varying from 2 to 6 alleles) in this study is an indication of considerable allelic variants per locus (genetic diversity present) among the tomato accessions under investigation (Moghaddam, and Alikhani, 2009).

A gene is said to be polymorphic if the frequency of one of its alleles is less than or equal to 0.95 or 0.99 (IPGRI and Cornell University, 2003). Results observed from allelic frequency analysis proved that all the 14 out of the 15 primers were polymorphic. No rare alleles (alleles with allelic frequencies of less than 0.005) were obtained. This was probably due to the genetic closeness of the genotypes studied. Gene diversity values of 0.61 on average were also observed. This demonstrates genetic polymorphism in the tomato germplasm studied.

Conclusions

Agglomerative hierarchical clustering and SSRs markers were effective in assessing varieties and molecular diversity within the tomato germplasm collection. The introduced tomato accessions including the local check used in the study were genetically variable and therefore clustered them in groups based on their close relationships or associations. The molecular markers grouped the genotypes into two (2) main clusters (A and B) with B having three sub-clusters (Fig. 7). Primers SSR1, SSR2, SSR7, SSR12 and SSR15 recorded the highest number of alleles detected per locus. SSR9 however, gave the highest PIC.

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