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Molecular Characterization of Three Cultivars of Tomato (*Lycopersicon Esculentum L.*) in South-West Nigeria Using SSR Markers

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Abstract**—** *Molecular characterisation of local tomato cultivars – Ibadan Local (IbL), Ife and JM94/46 (JM) were assessed using simple sequence repeat (SSR) markers. Out of ten SSR primer pairs used, three primer pairs were able to differentiate amplified genomic DNA of the cultivars. Unweighted Pair Group Method Using Arithmetic Average (UPGMA) cluster analysis of the data showed a close relationship between IbL and Ife with a genetic distance (GD) of 0.067; Ife and JM had GD of 0.2 and JM and Ife had GD of 0.25.*

Keywords**— Genetic Distance, Local cultivars, Nigeria, SSR Markers, Tomato.**

I. INTRODUCTION

The genetic analysis of relatedness between or within different species, populations and individuals is a prerequisite towards effective utilization and protection of plant genetic resources (Weising et al. 1995). The use of molecular markers in the characterization of much diversified materials offers a unique opportunity to define significant marker- trait associations of biological and agronomic interest; it also has proven to be a valuable tool in the evaluation of genetic variation both within and between species (Powell et al. 1996).

The genome of tomato plant is one of the most investigated plant genomes (Foolad 2007) and recent studies show that several researchers have characterized tomato varieties of interest using molecular markers. Random amplified polymorphic DNA (RAPD) marker studies in tomato has been conducted by El-Hady et al. (2010), Comlekcioglu et al. (2010), Naz et al. (2013), Mazzucato et al. (2008), Sharifova et al. (2013), Pal and Singh (2013), (Tabassum et al. 2013), Thamir et al. (2014) and Shah et al. (2015) while Amplified fragment length polymorphism (AFLP) marker studies in tomato was recently done by Berloo et al. (2008).

Simple Sequence Repeats (SSR) marker studies have recently been carried out by Benor et al. (2008), El-Awady et al. (2012), Korir et al. (2014) and Singh et al. (2014) .The high degree of polymorphism and the large number of bands obtained per assay shows that SSR is the most informative marker system for tomato genotyping for purposes of rights protection and for the tomato industry in general (Korir et al. 2014). SSR markers have the advantages of being co-dominant, reproducible, multiallelic, highly polymorphic, and assayable by PCR (Miskoska– Milevska et al, 2011).

Tomato fruits are a significant source of nutrition for substantial portions of the world's human population because this vegetable crop is widely cultivated and consumed extensively as both a fresh vegetable and concentrated processed products (Hamner and Maynard, 1942; Beecher, 1998). In tropical Africa, the area used for tomato cultivation is about 300,000 ha with an estimated annual production of 2.3 million tonnes; Nigeria is the largest producer accounting for 541,800 ha and an annual production of 2,143,500 tonnes (FAOSTAT 2014). Nigeria ranks 14th in the world in production, and 3rd in hectares of land cultivated (FAOSTAT, 2014) There is however paucity of documented work on diversity studies of Nigerian cultivars of tomato; such work would provide the background work for the application of modern biotechnology techniques in solving agricultural problems by providing new advances for the development and production of indigenous stress tolerant cultivars. The aim of this research was to analyze and characterize the genetic variability of some Nigerian Tomato cultivars.

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The choice of the tomato cultivars from South West Nigeria was based on agronomic studies carried out at the National Institute for Horticultural Research and Training (NIHORT) suggesting that Ibadan local (IbL) and Ife cultivars are farmer preferred varieties in the south-western part of Nigeria and are reported to be resistant to certain diseases and relatively high yielding (Badra *et al*., 1984; Anno-Nyako and Ladunni, 1984).

II. MATERIALS AND METHODS

2.1 Sample collection and preparation

This research work was carried out in the Central Biotechnology Laboratory at the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State. The tomato cultivars, Ibadan local (IbL), JM94/46 and Ife cultivars were collected as seedlings from the National Institute of Horticultural Research and Training (NIHORT), Ibadan, Oyo State and cultivated in a nursery bed for four weeks to produce fresh leaves.

2.2 Extraction of DNA

DNA extraction was carried out using modified Dellaporta (1983) procedure. Fresh leaves (0.15 – 0.2 g) from young tomato plants (3-4 weeks old) were harvested and ground freshly in liquid nitrogen with a plastic pestle in 1.5 ml eppendorf tube. 800 µl of extraction buffer (100mM Tris-HCl, pH 8.0, 50mM EDTA, pH 8.0, 500mM NaCl, 1% PVP) and 20 µl of 0.7% β-mercarptoethanol was quickly added and mixed until the tissues became dispersed in the buffer. Afterwards, 100 µl of 20% Sodium deodecyl sulphate (SDS) was added and mixed thoroughly for 1 min and incubated at 65°C in a water bath (GFL), mixing intermittently 5-6 times for 15 minutes. Samples were removed from 65°C and allowed to cool to room temperature (30 ± 2°C) before 300 µl of ice-cold potassium acetate was added and mixed by gently inverting 5-6 times and incubated on ice for 20 min. Samples were centrifuged (Eppendorf 5417C) at 14,837.76 g for 10 minutes and the supernatant was carefully transferred to new eppendorf tubes. 700 µl of ice-cold isopropanol was added and inverted gently 8-10 times, incubated at -80°C for 1 hr and then centrifuged 14,837.76 g for 10 minutes. The supernatant was tipped off ensuring the removal of the last drops of isopropanol. Pellets were re-suspended in 250 µl of high salt Tris-EDTA (TE) and 4 µl of 10 mg/ml RNase (Sigma) and incubated at 37°C for 30 min with constant gentle shaking. A 500 µl portion of ice-cold isopropanol was added, mixed by inverting 8-10 times, incubated at -80°C for 1 hr and centrifuged at 14,837.76 g for 10 minutes. Supernatant was tipped off removing last drops of isopropanol and then washed twice in 70% ethanol, centrifuging at 14,837.76 g for 10 min each time. Pellet was allowed to dry and 100 µl of sterile distilled water was added. Samples were stored at 4°C overnight to dissolve pellets. The concentration in ng/µl was measured at 260-280 nm with Nanodrop spectrophotometer (ND1000).

2.3 Polymerase Chain Reaction (PCR)

Ten SSR primer pairs (Suliman-Pollatschek *et al*., 2002) were used for the Polymerase Chain Reaction. PCR was carried out with Peltier thermal cycler-PTC200 using PCR conditions as described by Rajput *et al*. (2006). A 25 µl of PCR mix contained 2.5µl 10X reaction buffer (100mM Tris-HCl pH 9, 15mM $MgCl_2$, 500M KCl and 0.1% Gelatin), 3µl dNTPs (200 mM), 2µl of each forward and reverse primers 5 pica moles/ml primer, 1µl of 50 ng/ml genomic DNA and 0.8 U/ml *Taq* polymerase (Sigma) in addition to deionised water to complete the reaction mix. Only one DNA sample and both forward and reverse primer were added to any single reaction. The PCR programme used: one cycle (an initial denaturing step) at 94°C for 3 min; 35 cycles at 94°C for 1 min (denaturing); 55°C for 1 min; 72°C for 1 min 30 sec and one cycle (final extension) at 72°C for 7 min, kept at 4°C. The PCR amplification products were temporarily stored at -20°C. Electrophoresis of the amplified DNA products was carried on 3% agarose gel and 6% polyacrylamide gel for the determination of bands. The size of the alleles was determined by comparison with Hyper ladder V marker (Bioline) loaded on adjacent gel tracks.

Table.1: List of primers and sequence

S/N	SSR Repeat	Forward Primer 5' 3'	Reverse Primer 5' 3'
1	Tom 8-9 ATT7	GCA TTG ATT GAA CTT CAT TCT CGT CC	ATT TTT GTC CAC CAA CTA ACC G
2	Tom 11-28 CTT5/CT5	ATT GTA ATG GTG ATG CTC TTC C	CAG TTA CTA CCA AAA ATA GTC AAA CAC
3	Tom 31A-32A	AAT GTC CTT CGT ATC	CTC GGT TTT AAT TTT TGT

	TA11	CTT TCG T	GTC T
4	Tom 39A-40A AATT4	TAA CAC ATT CAT CAA AGT ACC	TTG CGT GAT CCA GTA AT
5	Tom 41-42TCC6	GAA ATC TGT TGA AGC CCT CTC	GAC TGT GAT AGT AAG AAT GAG
6	Tom 43-44TCC6	GCA GGA GAT AAT AAC AGA ATA AT	GGT AGA AGC CCG AAT ATC ATT
7	Tom 47-48 AT10	CAA GTT GAT TGC ATT ACC TAT TG	TAC AAC AAC ATT TCT TCT TCC TT
8	Tom 49-50 AT10	AGA AAA CTT TTT GAA TGT TGC	ATT ACA ATT TAG AGA GTC AAG G
9	Tom 55-56ATTT5	ATT TCT GTA ACT CCT TGT TTC	TGA CTT CAA CCC GAC CCC TCT T
10	Tom 57-58CT8	TCT AAG TGG ATG ACC ATT AT	GCA GTG ATA GCA AAT GAA AAC

2.4 Gel electrophoreses

2.4.1 Agarose gel electrophoresis of tomato genomic DNA

Using procedures as described by Rajput *et al*., (2006), 3% Agarose gel was prepared by weighing 4.5g of agarose powder and melting in 150 ml 1% TBE buffer (10.8g Tris-base; 5.5g boric acid; 20mM EDTA in 1 L) in a microwave oven (100 ^{o}C) until completely dissolved. The gel was allowed to cool slightly (about 40 ^{o}C) by continuous stirring on the magnetic stirrer (Thermolyne Cimarec 2) and then poured into the gel tank to set with the appropriate combs. 5 µl of gel loading dye was added to 5 µl of PCR product and spun down in the centrifuge to mix thoroughly. The samples were loaded on the gel; with Hyper ladder V marker (Bioline) loaded on adjacent gel tracks to determine the size of the alleles by comparison and allowed to run for 2-3 hr at 100 volts (Voltmeter EC 105). The gels were stained with 10 mg/ml ethidium bromide, visualized on a 302 nm UV transilluminator and photographed with a UVP bioimaging system (GDS-800).

2.4.2 Polyacrylamide gel electrophoresis (PAGE) of tomato genomic DNA

The long and short plates were washed until squeaky clean and wiped with ethanol. Long and short plates were treated with gel slick and 3µl bind silane in 95% ethanol respectively. When the plates dried (10 min) they were arranged on the gel caster. 600 µl of ammonium persulphate (NH_4SO_4) and 60 µl of temed were added to 60 ml of polyacrylamide solution and gradually poured between the plates before solidification. The comb was inserted and the plates clamped together and allowed to dry for about 1 hr. The clamp and comb were removed, the plates mounted on the gel ridge and the anode and cathode filled with 1X TBE (10.8g Tris-base; 5.5g boric acid; 20mM EDTA in 1 L) buffer to the lane levels and pre-ran for about 45 – 60 min at 1000 amps. A mixture of PCR product to bromophenol blue dye was prepared in the ratio 2:1 and denatured in the PCR machine for 5 min and immediately placed on ice. The power was disconnected to insert the comb and to quickly load the samples and allowed to run for 2 hr. The plates were separated and the short plates were placed in 200 ml acetic acid:1800 ml distilled water fixing solution with continuous shaking for 20 min. The plate was rinsed 2-3times with distilled water and transferred to staining solution consisting of 2 g of $AgNO_3$ in 2000 ml distilled water and 3 ml of 37% formaldehyde agitating well for 30 min. It was rinsed briefly in ultrapure water (5-10 sec) and transferred to 1 L of chilled developing solution consisting of 60g of sodium carbonate in 2000 ml distilled water with 3 ml of 37% formaldehyde and 40µl of sodium thiosulphate. The plate was agitated very well in the developing solution and when the first bands were visible; the fresh solution was replaced with the remaining 1 L and agitated till all the bands were visible. The plate was dipped into the fixing solution shaking for 2-3 min to stop the reaction and then rinsed in ultrapure water twice while shaking. The plate was allowed to dry by leaving at room temperature.

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2.5 Molecular Characterisation of the three local tomato cultivars

Characterization/amplification of the three tomato cultivars with simple sequence repeat (SSR) markers and the genetic and phylogenic data analysed using NTSYS (Applied Biostatistics Inc. version 2.0) software by the clustering method of the Unweighted Pair Group Method using Arithmetic Average (UPGMA).

2.6 Genetic similarity estimation and cluster analysis

All distinct DNA fragments were scored as present {1} or absent {0} for each of the markers. The genetic similarity (GS) estimates between two cultivars *i* and *j* was estimated following the methods of Nei and Li (1979), which is defined as:

(1) $Sij = 2Nij / (Ni + Nj)$

Where *Nij* is the number of bands present in the cultivars *i* and *j*, and *Ni* and *Nj* representing the number of bands present in cultivar *i* and *j*, respectively.

For phylogenetic analysis, only data from the polymorphic SSR loci were subjected to NTSYS statistical software. The 3 cultivars were clustered based on the estimated genetic distance, and the phylogenetic analysis was carried out with the clustering method of the Unweighted Pair Group Method Using Arithmetic Average (UPGMA).

III. RESULTS

3.1 Estimation of genetic similarity

Genetic similarity among the cultivars was deduced from the banding patterns on the agarose and polyacrylamide gel electrophoreses in Fig. 1 and Fig. 2 which showed polymorphism among IbL, JM94/46 and Ife cultivars with primers T3, T8 and T10. Monomorphic bands were disregarded. The primers were able to differentiate the three cultivars by the presence or absence of amplified bands. Polymorphism for T10 was between 150 – 200 bp for T10; and 200 – 250 bp for T3 and T8. A total of 35 bands were obtained with 10 SSR primer pairs (TABLE 4) out of which 10 were polymorphic. Genetic similarity estimates between IbL (1) and Ife (3) was highest at 0.90. JM showed the least similarity to the other two cultivars at 0.65. The presence or absence of bands at any loci differentiates one cultivar from the other and were statistically analysed by UPGMA cluster analysis (Nei and Li, 1979) to obtain the dendogram and genetic similarity coefficients shown in Fig. 3 and TABLE 2 respectively.

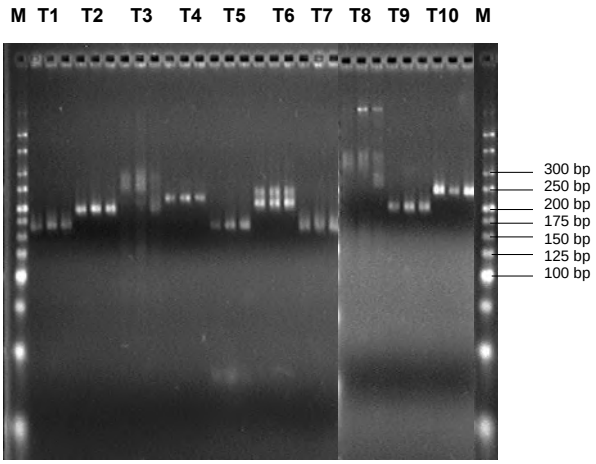

Fig.1: Agarose gel showing the alleles using 10 SSR primer pairs (T1-T10), M-Hyper ladder V to determine the allele sizes. Cultivars are arranged: Ibadan local (IbL), JM94/46 (JM), Ife

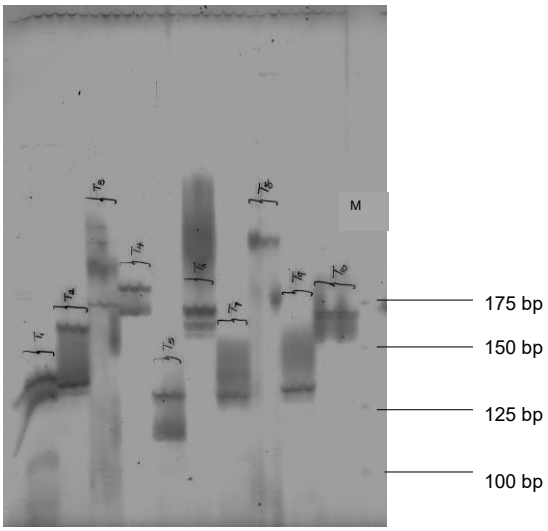

Fig. 2: PAGE gel showing the alleles using 10 SSR primer pairs (T1-T10), M-Hyper ladder V marker to determine the allele sizes. Cultivars are arranged: Ibadan local (IbL), JM94/46 (JM), Ife

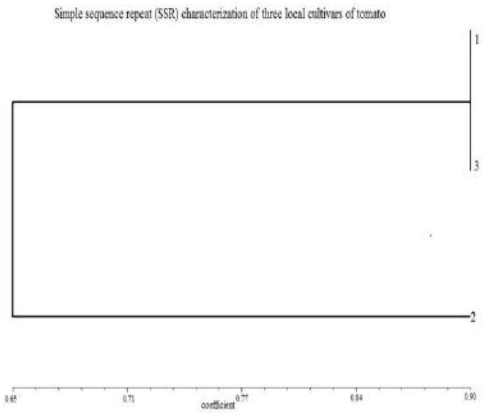

Fig.3: Dendogram of simple sequence repeat (SSR) primers for characterization of three tomato cultivars. 1-IbL; 2-JM; 3-Ife

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Genetic distances obtained using three SSR markers constructed by UPGMA clustering of Nei and Li (1979).

Table.2: Genetic similarity coefficients among three tomato cultivars

	Line1	Line2	Line3
Line 1	0.0000		
Line 2	0.2000	0.0000	
Line 3	0.0667	0.2500	0.0000

Line1-IbL; Line2-JM; Line3-Ife

GD 01-02 = 0.2; 01-03 = 0.0667; 02-03 = 0.25

GD = genetic distance

3.2 Primer evaluation/Characterization of primers

Out of the ten primer pairs (TABLE 1) used in the characterization of the three tomato cultivars, three of them were polymorphic i.e. primers T3, T8 and T10 (TABLES 3 and 4). The polymorphic information content (PIC) of the polymorphic markers was evaluated using the formula:

(2) PIC = 1 – ∑ pi2

Pi = frequency of ith allele (Weir, 1990)

pi2 = (relative frequency)2 = total sum of frequency/each frequency

From the data, Tom 57-58 (T10) had three alleles with bands either present or absent at each locus between the range of 150-175bp. It had the highest polymorphic inforation content (PIC) value of 0.816 (81.6 %). Tom 31A-32A (T3) had five alleles with bands either present or absent between 140-300bp and (PIC) value of 0.778 (77.8 %). The least PIC value of 0.375 (37.5 %) was recorded for Tom49-50 (T8) with two alleles and bands present or absent between 160-350bp. Average PIC value was calculated to be 0.656 (65.6 %).

Table.3: Polymorphic information content (PIC) of polymorphic markers

Primer/ Cultivar	**Ibadan local**	**JM94/46**	**Ife**	**Sum of freq**	**freq(i)**	**{freq(i)}^{2}**	**PIC**	
T3A	1	1	1	3	0.2500	0.063		
T3B	0	1	0	1	0.0830	0.007		
T3C	1	0	1	2	0.1660	0.028		
T3D	1	1	1	3	0.2500	0.063		
T3E	1	1	1	3	0.2500	0.063		
				12		0.222	0.778	
T8A	0	0	1	1	0.2500	0.063		
T8B	1	1	1	3	0.7500	0.563		
				4		0.626	0.375	
T10A	0	1	0	1	0.143	0.021		
T10B	1	1	1	3	0.429	0.184		
T10C	1	1	1	3	0.429	0.184	0.816	
				7				
Average PIC value								0.656
Highest								0.816
Lowest								0.375

Table.4: Characteristics of Polymorphic SSR markers used in the study

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S/N	LD.	SSR Name/ Repeat	Forward Primer 5' 3'	Reverse Primer 5' 3'	No of Alleles	Allele size (bp)	PIC
1	T3	Tom 31A-32A TA11	AAT GTC CTT CGT ATC CTT TCG T	CTC GGT TTT AAT TTT TGT GTC T	5	140-300	0.778
2	T8	Tom 49-50 AT10	AGA AAA CTT TTT GAA TGT TGC	ATT ACA ATT TAG AGA GTC AAG G	2	160-350	0.375
3	T10	Tom 57-58 CT8	TCT AAG TGG ATG ACC ATT AT	GCA GTG ATA GCA AAT GAA AAC	3	150-175	0.816

IV. DISCUSSION

Molecular markers are an effective tool for efficient selection of desired agronomic traits because they are based on the plant genotypes and also are independent of environmental variations (Sunilkumar et al, 2016).

4.1 Molecular Characterisation with simple sequence repeat (SSR) markers

Ten (10) SSR primer pairs were used for molecular characterisation of three Nigerian cultivars of tomato. This was carried out to determine their genetic similarity and variability. The primer pairs were used to amplify specific segments of the tomato genome in order to generate the relevant data. Three (3) of the ten primer pairs amplified polymorphic segments of the three tomato cultivars and the data obtained was used to estimate the genetic similarity (TABLE 2) and to determine the genomic cluster of the cultivars on the phylogenic tree (Fig. 3). The data was also used to determine the polymorphic information content (PIC) of the primers (markers) (TABLE 3).

4.2 Agarose gel electrophoresis

Fig. 1 shows the resolution of the amplified alleles of the tomato DNA on agarose gel electrophoresis. The allele sizes ranged between 140-350 bp.

4.3 Polyacrylamide gel electrophoresis (PAGE)

Although the popularity of PAGE gels is declining, mainly due to the drudgery of the method and to comparable efficiency and simplicity of agarose gel; they usually give a higher resolution than agarose gels because the amplified DNA is denatured before running them on PAGE gel. Fig. 2 is the PAGE gel of the three tomato cultivars. The allele sizes ranged between 100-350 bp. The alleles are more distinct and data easier to record with PAGE gel. Due to the close genetic relationship among modern tomato cultivars and their narrow genetic base (Alvarez et al., 2001; Zhang et al., 2003), PAGE gels could be more efficient in distinguishing between tomato cultivars.

4.4 PIC of primers

The highest PIC was recorded for primer pair T10 with PIC value of 0.816, and lowest was 0.375 for primer pair T8. The PIC value for T3 was also high at 0.778. Average PIC value of the three polymorphic primers was 0.656. With the value of 1.0 being the highest/max, the two primers, T3 and T10 are highly polymorphic. The highest number of alleles was recorded with primer T3. García-Martínez et al. (2006) reported PIC values between 0.035 and 0.775 for tomato germplasm evaluated with amplified fragment length polymorphism (AFLP) while Bredemeijer et al. (2002) obtained PIC values of 0.40 evaluating 500 varieties of tomato with SSR markers. These results may suggest that highly polymorphic markers are ideal to conduct assessments aimed at understanding the genetic diversity of plant crops.

4.5 Genetic similarity/diversity of cultivars

The genetic distance (GD) among the three cultivars as estimated showed the highest GD between JM and Ife (0.25); least GD was between IbL and Ife (0.0667) and between IbL and JM (0.2). These values show that the cultivars are all closely related. Close genetic relationship has been reported in tomato cultivars due to lack of variability that was ascribed to the self-pollinating nature of modern tomato cultivars combined with their narrow genetic base (Alvarez et al., 2001; Zhang et al., 2003). Also, the genetic similarity estimated according to SSR data suggests the potential of SSR markers in discriminating among plants of close or distant genetic backgrounds (El-Awady et al, 2012). This study shows that the genetic similarity between the three tomato

cultivars suggests the need for more analysis using tomato varieties across the geo political zones of Nigeria for the purpose of maintaining the tomato germplasm, understanding its genetic diversity and as a prerequisite for effective breeding programme.

V. CONCLUSION

The SSR marker system is useful for studying genetic diversity among tomato inbred lines collected from diverse geographical locations. The combination of polymorphism and the large number of bands obtained per assay shows that SSR is the most informative marker system of tomato genotyping. The work of Smulders *et al*. (1997), Bredemeijer *et al*. (2002), He *et al*. (2003), Frary et al. (2005), Garcia- Martinez *et al*. (2006) and Song *et al*. (2006) confirmed the utility of SSRs for studying genetic diversity and variability in the genus *Solanum* and for selecting tomato cultivars.

This study showing the genetic similarity between the three tomato cultivars suggests the need for more analysis using tomato varieties across the geo political zones of Nigeria for the purpose of maintaining the tomato germplasm, understanding its genetic diversity and as a prerequisite for effective breeding programme. More efforts should be directed at preserving our indigenous germplasm for research and economic purposes. It is also very essential to carry out the characterization of cultivated and economically useful; as well as neglected and underutilized indigenous genetic resources in the Nigerian eco-system.

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