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AFLP MARKERS BASED DIVERSITY ANALYSIS AMONG WILD RELATIVES OF TOMATO COLLECTED FROM CHHATTISGARH REGIONS OF INDIA

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ABSTRACT
The cultivated Tomato (*Solanum lycopersicum*) is the second most consumed vegetable worldwide and a well studied crop species in terms of genetics, genomics, and breeding. For the current study wild relatives consisted of cherry and medium sized tomato fruits of 10 tomato genotypes were collected from different regions of Chhattisgarh in order to find out the genetic diversity among these. AFLP analysis revealed that, a total of 2209 reliable polymorphic bands were observed in the 9 AFLP gels varied from 128 to 417, of which only 3 was monomorphic across all 10 genotype. The average no of polymorphic bands present across genotype per primer combination varied from 2.11- 5.48. All the primer combination used in this study has >0.800 PIC value and ranged from 0.813 (ESA14 Vs MSA12) to 0.898 (ESA13 Vs MSA14). The Jaccard's similarity coefficient for AFLP data set varied from 0.13 to 0.60. The 10 genotype formed 2 major clusters at 13 % similarity level. The lowest similarity (0.13) was between genotypes Raipur cherry and Janjgir long which were the most divergent while the highest similarity (0.72) was found between the genotypes jashpur cherry and Janjgir cripping. This study demonstrated that AFLP markers are effective for obtaining unique fingerprints of, and assessing genetic diversity among, tomato wild relatives.

INTRODUCTION

The cultivated Tomato (*Solanum lycopersicum*) is the second most consumed vegetable worldwide and ranking first in the world for vegetables, accounts for 14% of world vegetable production (over 100 million metric tons/year $ 1.6 billion market; (Food and Agriculture Organisation (FAO, 2010). It also a well studied crop species in terms of genetics, genomics and breeding. The cultivated tomato was originally named *Solanum lycopersicum* by Linnaeus (Linnaeus, 1753).

The major goals of tomato breeders (higher productivity, better tolerance to biotic and abiotic stresses and increased sensory and health value of the fruit) require a good comprehension and management of tomato genetic resources diversity. Due to its Latin American origin and related domestication history, cultivated tomato has faced several bottlenecks over ages. This led to a drastic reduction of its genetic diversity. Explorations of tomato centre of origin permitted major advances in the characterization of its diversity. In parallel, ex situ plant conservation initiatives bloomed, ensuring the collection and conservation of landraces and wild species through development of seed banks. Thus, unraveling the genetic potential of tomato's wild relatives for breeding purpose emerged (Bauchet and Causse, 2012). In addition to the cultivated species of tomato there are eight related wild species, including *L. pimpinellifolium* (Jusl.) Mill (currant tomato). Cultivated tomato is well-known for its low level of DNA-polymorphism. Less than 10% of the total genetic diversity in the *Lycopersicon* gene pool is found in *L. esculentum* (Rick, and Butler, 1956).

Genetic markers are the useful source for diversity analysis and breeding programmes of any species (Mallikarjuna *et al.*, 2012). DNA fingerprinting is an ideal tool for assessing genetic diversity and aiding cultivar identification, because it measures differences between individual plants or genotypes at the DNA level without being obscured by complex pedigree records, environmental conditions, or epistatic and pleiotropic effects (Hongtrakul *et al.*, 1997; Rasmusson and Phillips 1997; Seefelder *et al.*, 2000). Various DNA marker systems, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs or microsatellites), have been successfully used for variety identification and genetic diversity studies in crop species (e.g., Miller and Tanksley 1990; Williams and St.Clair 1993; Hongtrakul *et al.*, 1997; Barrett and Kidwell 1998; Lombard *et al.*, 2000; Archak *et al.*, 2002; He *et al.*, 2003).

AFLP is a highly reproducible marker technique based on the detection of genomic restriction fragment by PCR amplification, and can be used for DNA of any origin or complexity. Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. that can be used to

efficiently detect DNA polymorphisms (Vos *et al.*, 1995; Jones *et al.*, 1997). AFLPs have been used in crop species for germplasm fingerprinting and genetic diversity evaluation (Hill *et al.*, 1996; Maughan *et al.*, 1996; Hongtrakul *et al.*, 1997; Lombard *et al.*, 2000; Barrett and Kidwell, 1998; Koopman *et al.* 2001). In tomato, a high-density AFLP map has been constructed using an interspecific population (Haanstra *et al.*, 1999). However from best of our information, less number of studies using AFLPs to assess genetic diversity among tomato cultivars has been published. In order to estimate the genetic diversity of tomato wild relatives of Chhattisgarh region the objective of current study is to find out variability among different wild relatives of tomato through AFLP markers.

MATERIALS AND METHODS

The experimental material consisted of 10 wild relatives of tomato were collected from the northern hills (Sarguja) and plain regions (Raipur, Durg, Bilaspur, Jashpur, Janzgir) of Chhattisgarh, Which were used to work out the genetic diversity among these.

In the current study Genomic DNA was isolated from the small pieces of leaf tissues (~ 1 cm^2) from four-week old plants of the tomato genotypes by CTAB (Cetryl trimethyl ammonium bromide) method previously used by (Murry and Thompson., 1980) with little modification as reported by (Ginwal and Mittal., 2010) for removing the phenolics and RNA. Before starting, add B-merceptaethanol (20 μL/20 ml Buffer), 8M Lithium chloride (300 μL/ 1000 μL) and 4% poly vinyl pyrollidone (PVP) to CTAB extraction buffer. and isolated DNA were resuspended in 25μL TE buffer and were keep at -20Úc for further use. DNA was quantified by NanoDrop (ND-1000 spectrophotomer V3.5) spectrophotometer followed by dilution to get a final concentration 25ng DNA/μL.

AFLP based Fingerprinting of Collected Tomato Wild Relatives

AFLP analysis followed the protocol of Vos *et al.,* (1995) with minor modifications. In this study, we used both *Eco*RI and *Mse*I restriction enzymes, based on prior polymorphism information from AFLP mapping in tomato (Haanstra *et al.,* 1999). Samples containing on an average 600 ng /μL volume of genomic DNA from each isolate were digested with 5.0 units of *Eco*RI and 1.0 units of *Mse*I (New England Biolabs, Beverly, MA). Nine different sets of AFLP primers (ESA11 Vs MSA11, ESA14 Vs MSA12, ESA12 Vs MSA11, ESA12 Vs MSA12, ESA12 Vs MSA13, ESA13 Vs MSA13, ESA13 Vs MSA14, ESA12 Vs MSA14, ESA14 Vs MSA14) were used for PCR based DNA fingerprinting analysis in order to identify polymorphism between wild relatives of Tomato spp. AFLP pre-selective and selective reactions were performed where DNA samples of 10 wild Tomato spp were cleaved with restriction enzymes *Eco*RI and *Mse*I at 37ºC for 5 hrs followed by ligation with *Eco*RI adapter F [17 bases] (5′-CTC GTA GAC TGC GTA CC -3′); *Eco*RI adapter R [18 bases](5′-AAT TGG TAC GCA GTC TAC -3′)and *Mse*I adapter F [16 bases] (5′-GAC GAT GAG TCC TGA G-3′); *Mse*I adapter R [14 bases] (5′-TAC TCA GGA CTC AT -3′) AFLP adaptors at temperature 22ºC for 6 h. For pre-selective amplification, 4 μL of the 20-fold diluted ligation mixture was amplified for 20 cycles of 30s at 94ºC, 1 min at 56ºC and 1 min at 72ºC, using the Gradient Palm Cycler, Corbett Life Science, Sydney, Australia. EcoRI primer 1 [17 bases] (5′- GAC TGC GTA CCA ATT CA-3′); MseI 1 primer (5′-GAT GAG TCC TGA GTA AA-3′) were used as preselective amplification primer. Products from the pre-selective amplification were diluted 20-fold and used as templates for the selective amplification. For selective amplification and subsequent detection on the sequencing gel (Bio-RAD), *Eco*RI and *Mse*I primers with one selective nucleotides at their 32 end were used in different combinations (Table 1). Amplification conditions consisted of an initial denaturation step at 94ºC for 2 min followed by 10 cycles at 94ºC for 20 s, primer annealing consisted of a 1ºC per cycle step-down starting at 66ºC for 30 s and 72ºC for 2 min for DNA extension, followed by 20 cycles of 94ºC for 20 s, 56ºC for 30 s, and 72ºC for 2 min, and a final hold at 60ºC for 30 min (GeneAmp 9700 PCR system). The products from this selective amplification were prepared for analysis by diluting 30-fold in Loading Solution. Fragment separation and detection was performed with the Sequencing gel Bio RAD system, using denaturating gel electrophoresis.

Cluster analysis

The AFLP autoradiographs were scored for the presence (1) or absence (0) of all polymorphic bands generated in a 12 x 156 binary data matrix from nine primer combinations. All subsequent data analysis was performed using NTSYSpc version 2.0 (Rohlf, 1997). Pair wise genetic similarities based on Jaccard's (1908) coefficient were applied to the AFLP data-sets. The similarity matrices were subjected to sequential agglomerative hierarchical nested (SAHN) clustering using UPGMA (Unweighted pair-group method with arithmetic averages) in NTSYS-pc software version 2.0. Jaccard similarity coefficient was calculated as

$$\frac{a}{(n-d)}$$

for each pair of cultivars, where a is the number of loci for which the band is present, d is the number of loci for which the band is absent and n is the total number of loci. The results were presented in the form of dendrogram. The dendrogram were visualized and edited using coral draw version 13.

RESULTS

The purpose of this study is to explore the utility of AFLPs to elucidate phylogenetic relationships of wild tomatoes. A total of 2209 reliable polymorphic bands were observed in the 9 AFLP gels, of which only 3 was monomorphic across all 10 genotype.

This current study revealed that large number of bands observed demonstrates that AFLP analysis is a robust and efficient method for detecting differences between 10 tomato wild relatives.

The number of polymorphic bands per primer varied from 128 to 417. ESA12 Vs MSA14 (417), ESA13 Vs MSA14 (206), ESA13 Vs MSA13 (207), ESA14 Vs MSA14 (286), ESA11 Vs MSA11 (106), ESA12 Vs MSA12 (259), ESA14 Vs MSA12 (351), ESA12 Vs MSA11 (128), ESA12 Vs MSA13 (249) (Table 1). The average no of polymorphic bands present across

Table 1: List of selected informative AFLP primer combination, average number of bands, number of polymorphic bands and their Polymorphic Information Content (PIC)

Primer combination	Average no of band Across genotype	No. of Polymorphic Bands	PIC Value
ESA11 Vs MSA11 = E-AAA/M-CAC	2.94	106	0.841
ESA14 Vs MSA12 = E-AAG/M-AAC	5.48	351	0.898
ESA12 Vs MSA11 = E-AAC/M-CAC	4.00	128	0.863
ESA12 Vs MSA12 = E-AAC/M-AAC	3.27	259	0.879
ESA12 Vs MSA13 = E-AAC/M-ATT	2.11	249	0.880
ESA13 Vs MSA13 = E-AAT/M-ATT	3.23	207	0.853
ESA13 Vs MSA14 = E-AAT/M-CTT	2.90	206	0.813
ESA12 Vs MSA14 = E-AAC/M-CTT	3.79	417	0.888
ESA14 Vs MSA14 = E-AAG/M-CTT	4.93	286	0.893

Table 2: Genetic similarity matrix based on 9 different combinations of AFLP markers among 10 Tomato genotypes

	Janzgir Cherri	Raipur Cherri	Durg Cherri	Jashpur Medium	Sarguja	Janzgir Long	Durg Medium	Bilaspur Cherri	Jashpur Cherri	Janjgir Criping
Janzgir Cherri	1.00									
Raipur Cherri	0.18	1.00								
Durg Cherri	0.08	0.14	1.00							
Jashpur Medium	0.44	0.17	0.11	1.00						
Sarguja	0.13	0.19	0.17	0.15	1.00					
Janzgir Long	0.28	0.13	0.16	0.32	0.22	1.00				
Durg Medium	0.27	0.21	0.07	0.31	0.18	0.19	1.00			
Bilaspur Cherri	0.25	0.16	0.14	0.30	0.22	0.37	0.24	1.00		
Jashpur Cherri	0.31	0.19	0.14	0.37	0.24	0.43	0.26	0.58	1.00	
Janjgir Criping	0.30	0.16	0.12	0.31	0.19	0.42	0.24	0.51	0.60	1.00

genotype per primer combination varied from 2.11- 5.48 (Table 1). All the primer combination used in this study has >0.800 and ranged from 0.813 (ESA14 Vs MSA12) to 0.898 (ESA13 Vs MSA14) (Table 1).

Figure 1 shows the variation in leaf morphology along with AFLP picture. The Jaccard's similarity coefficient for AFLP data set varied from 0.13 to 0.60. The 10 genotype formed 2 major clusters at 13 % similarity level (Fig. 2). The major cluster A consisted of Janzgir Cherri, Jashpur medium, Janzgir long, Bilaspur Cherri, Jashpur Cherri, Janzgir criping, Durg medium, Raipur Cherri, and Sarguja. Major clusters A showed sub cluster at 19 % similarity level with A1 consisting of seven genotypes and A_2 consisting 2 genotypes. In the cluster A_1 3 genotypes belonged to Janzgir location, 2 genotypes belonged to Jashpur location, 1 genotype belonged to Bilaspur location and 1 genotype belonging to Durg location. The clusters A_2 consisted of two genotypes, belonging to Raipur and Sarguja locations. A_1 was sub clustered at 25 % similarity level to A_{11} and A_{12}. A_{11} consisting of 5 genotypes which showed high similarity were as A_{12} consisted of one genotype. In major cluster A, two genotypes Jashpur Cherri and Janzgir cripping were most similar with similarity coefficient of 60%. The cluster B consisted of only one genotype belonging to Durg location. The genetic similarity matrix reveals that the moderate level of similarity ranged from 0.13 to 0.60. The lowest similarity (0.13) was between genotypes Raipur cherry and Janjgir long which were the most divergent while the highest similarity (0.60) was found between the genotypes 'jashpur cherry and Janjgir cripping (Table 2).

DISCUSSIONS

Genetic diversity is characteristics of ecosystem and gene pools that describes an attribute which is commonly held to be advantageous for survival.The estimates based on morphology are not very reliable, on the other hand molecular markers are considered to provide the best estimates of available genetic diversity since these are independent of environmental factors (Tanksley *et al.*, 1989; Joshi *et al.*, 2013). Without genetic diversity it becomes difficult for a population to adapt to environmental changes creating a static population (Denis *et al.*, 2005). Creation of genetic variation and then selection of suitable genotypes is one of the common ways that can assist in crop improvement. It is becoming easier to enhance the exploitation of the germplasm and have great potential to identify the structure of genetic diversity within and among accessions, which is important for optimization of collections; the planning of seed regeneration and the successful implementation of prebreeding approaches (Yediay *et al.*, 2010; Shah *et al.*, 2015). Developmental breeding is now accepted as an important add-on to plant breeding, to introduce new traits from non-wild relatives, notably for abiotic stress (FAO, 2010). DNA fingerprinting is valuable analysis due to its high sensitivity and ability to distinguished close related genotypes. Various types of DNA markers studies have been carried out to estimate genetic diversity among different tomato genotypes and phylogenetic relationship among different tomato cultivars. AFLP is a highly reproducible marker technique that can be used to efficiently detect DNA polymorphisms. AFLP-based fingerprinting was effective for revealing DNA polymorphisms for fingerprinting and assessing genetic relationships among tomato cultivars (Vos *et al*,. 1995; Jones *et al.*, 1997). Tomato is heavily consumed in all over the world but the farmers suffer heavy losses due to biotic and abiotic stresses, so there is urgent need to develop tomato cultivars adapted to local agroclimatic conditions of

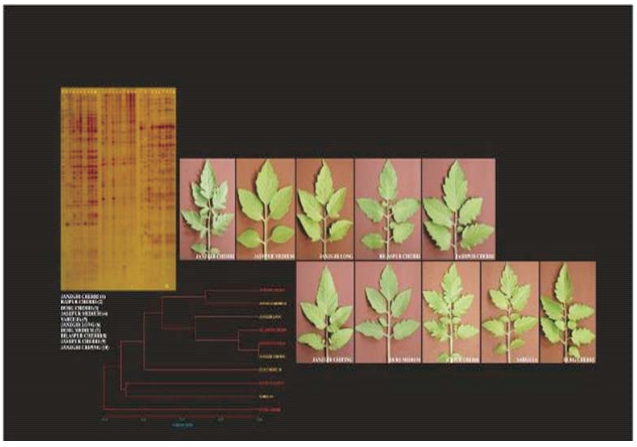

Figure 1: Variation in leaf morphology and genetic relationship inferred through UPGMA clustering of AFLP primer generated binary matrix for different genotypes of tomato analyzed using jaccard (1908) similarity coefficient by NTSYS programme

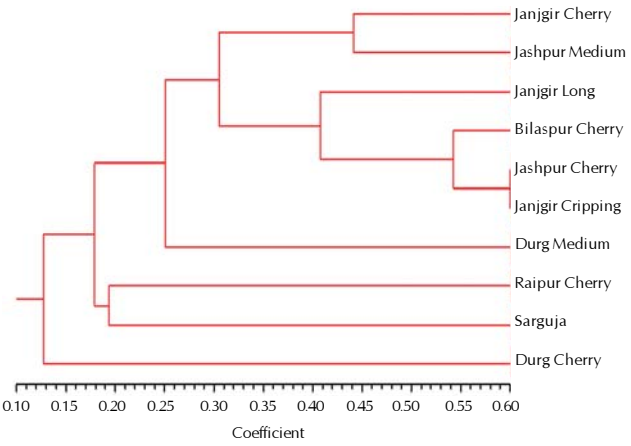

Figure 2: Genetic relationship inferred through UPGMA clustering of AFLP primer generated binary matrix for different genotypes of tomato analyzed using Jaccard (1908) similarity coefficient by NTSYS programme

Chhattishgarh taste preference of the local consumers. For this, there is need to characterize the tomato germplasm, landraces and wild relatives available in Chhattisgarh both at genotypic and phenotypic level in order to use them in breeding program effectively. In the current study Using a nine primer combinations, we detected AFLP fingerprints unique to 10 tomato wild cultivars (Fig.1). The results showed that wild relatives of tomato exhibited fingerprints that grouped them together in same cluster irrespective of their location except Durg cherry which was present in different cluster. The polymorphism level in the current study of AFLP based diversity analysis showed moderate to low level of similarity among genotypes which is quite similar to previous molecular diversity studies on cultivated tomato, whether it was based on RAPD (Williams and St. Clair, 1993), RFLP (Miller and Tanksley, 1990) SSR (Bredemeijer *et al.,* 2002; He *et al.,* 2006 or AFLP (Berloo *et al.,* 2008; Park *et al.,* 2004) and also in AFLP studies of cultivars in other self-pollinated crop species (e.g., wheat, Barrett and Kidwell, 1998; lettuce, Hill *et al.,* 1996). The ability of AFLPs is to uniquely identify closely and distantly related *spp* was also confirmed by the differentiation of wild relatives of tomato genotypes. The observed grouping patterns within major clusters (Figs. 1 and 2) are likely due in part to relatedness among the wild relatives within a node, but since most cultivar pedigrees were not available, it was not possible to test this hypothesis.

A study by Sharifova *et al.,* 2013 on Random Amplified Polymorphic DNA (RAPD) on 19 Azerbaijan tomato genotypes, both cultivars and local populations and he revealed low genetic similarity among evaluated genotypes ranged from 0.188 to 1.000 support our findings. This low level to medium level of similarity shows that the cherry, medium and long tomato wild relatives of Chhattisgarh regions are quite different in their genetic background. The moderate degree of similarity between jashpur cherry and Janjgir cripping may be due to the dry area and close geographical location.

The majority (90%) of the pair-wise Jaccard similarity coefficients below 0.5 (Fig. 2), which is quite opposite the result obtained by Park *et al.*, 2004 where, the majority (72%) of the pair-wise Jaccard similarity coefficients exceeded 0.5 suggesting that, many of the tomato wild relatives are members of a more diverse geographic origins.

Park *et* al., 2004 reported less number of polymorphic bands in AFLP analysis of 74 tomato cultivars, shah *et al.,* 2015; Sharifova *et al.,* 2013; Kulakarni and Deshpandey *et al.,* 2006 reported less number of polymorphic bands in RAPD analysis, however in our present study, comparatively higher number of polymorphic bands (128-417) detected its clearly indicated that AFLP fragments are highly polymorphic and particularly informative in the estimation of genetic diversity of tomato wild relatives than other molecular markers. The higher level of PIC content of set of AFLP markers used in this study support and prove its higher degree of usefulness in molecular marker based diversity analysis.

This study should be useful both for identification of its replica and expansion of core collection in the gene banks. Low levels of genetic similarity among the tomato wild relatives indicates that near geographical difference need not show difference and has fare chance to have very close similarity to cultivated variety. In the post genomic era of sequencing, helps to better characterization of its genetic diversity.

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