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Molecular characterization and diversity analysis in chilli pepper using simple sequence repeats (SSR) markers

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India is considered to be the secondary center of diversity of chilli pepper, especially of *Capsicum annuum*. Simple sequence repeats (SSRs) are the most widely used marker system for plant variety characterization and diversity analysis especially in cultivated species which have low levels of polymorphism. The diversity analysis of 64 chilli pepper accessions, mostly of Indian origin, was performed using 50 SSR markers. Twenty seven (27) polymorphic primers amplified a total of 75 alleles with an average of 2.78 alleles per locus. Maximum of four alleles were amplified by the primer AVRDC PP 32. The polymorphic information content (PIC) values ranged from 0.39 (AVRDC PP 138) to 0.78 (AVRDC PP 18), with an average of 0.59. Based on the PIC values, primers AVRDC PP 18 was found to be the most informative (0.78), followed by the primers AVRDC PP 32 (0.69) and AVRDC PP 03 (0.66). Using the given set of primers, it was possible to characterize all but two pairs of accessions from each other. The analysis allowed grouping of the test germplasm into nine clusters. Based on diversity analysis, genotypes were identified for developing mapping populations, produce heterotic F_1 hybrids and attempt crosses for genetic improvement of the crop.

Key words: Capsicum, genetic diversity, molecular characterization, simple sequence repeats (SSR) markers.

INTRODUCTION

Chilli pepper (*Capsicum annuum* L.) (Solanaceae) has a chromosome number 2n=2x=24. It is indigenous to South America and was first introduced in India from Brazil by Portuguese towards the end of 15th century (Basu and Krishna, 2003). Pepper is an often cross pollinated crop and, therefore, exhibits wide variability for different qualitative and quantitative traits (Tanksley, 1984). There are five cultivated species of peppers including *Capsicum annuum* L, *C. frutescens*, *C. chinense*, *C. pubescens* and *C. baccatum* (Heiser and Smith, 1957). India is considered to be the secondary center of diversity for chilli (IBPGR, 1983), especially of *C. annuum*, the most

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Abbreviations: AVRDC, Asian Vegetable Research and Development Center; **DUS,** distinctness, uniformity and stability; **IBPGR,** International Board for Plant Genetic Resources; **UPGMA,** unweighted pair group method with the arithmetic averaging; **PIC,** polymorphism information content.

important cultivated species. North-Eastern states are home to the genetic variability where several interspecific hybrids/derivatives were originated, among which Naga King is one of the world's hottest peppers. Over the years, chilli has become an important commercial crop of India and the country is currently the leading producer, consumer and exporter of peppers in the world. Although chilli pepper is cultivated almost throughout the country, Andhra Pradesh alone accounts for 25% of the total area and 40-50% of the total national production. In the world trade, India contributes about 25% of the total global pepper exports (Anonymous, 2011).

Genetic resources are the most valuable and essential basic raw material to meet the current and future needs for genetic improvement of any crop. Characterization of the germplasm is important for its identification and registration with the competent authority for plant variety protection. Conventionally, morphological markers called descriptors were used for varietal identification and genetic diversity analysis in plants that demands collection of extensive data at different locations. However, the level of polymorphism for morphological characteristics in elite germplasm is sometimes too limited and inadequate to allow for variety/genotype discrimination (Geleta et al., 2005). The traditional method of distinctness, uniformity and stability (DUS) testing is time-consuming and expensive, requiring large areas of land and skilled personnel, and is often subjective due to environmental influences (Singh et al., 2004). Further, taxonomy of the genus *Capsicum* is confusing and sometimes it is difficult to identify an accession using only subjective morpho-agronomic data (Costa et al., 2006).

In the last decade or so, molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) have been developed for pepper (Jang et al., 2004; Kang et al., 1997, 2001; Lee et al., 2004; Lefebvre et al., 2001; Moon et al., 2003; Paran et al., 1998; Prince et al., 1992). These markers have proven to be very useful in assessing genetic diversity and phylogeny, characterization of germplasm and detection of duplicates, parental verification in crosses, gene tagging in marker assisted breeding and gene cloning in genetic transformation (Costa et al., 2006). The application of RFLPs for genetic diversity is limited because it requires the use of radioactivity and is labour intensive (Nahm et al., 1997). RAPDs and AFLPs identify only dominant alleles and are sensitive to PCR amplification. The working group on biochemical and molecular techniques of UPOV has identified SSR markers as the most widely used marker system for plant variety characterization (UPOV-BMT, 2002). The SSR markers are especially suitable for diversity analysis in cultivated species which have low levels of variation as detected by other types of markers and have also been used in successful prediction of heterosis and performance of F_1 hybrids from morphological similarity of their parents (Geleta et al., 2004). The present investigation was undertaken to characterize and give robust genetic diversity estimates in cultivated chilli peppers using SSR markers.

MATERIALS AND METHODS

The experiment was conducted at the Vegetable Research Farm and the Vegetable Breeding Laboratory of the Department of Vegetable Science, Punjab Agricultural University, Ludhiana-141004, India during the years 2011 and 2012.

Experimental material and the SSR markers

The experimental material comprised 64 germplasm accessions. Majority of the accessions (49) belonged to the indigenous sources and the remaining (15) to the exotic sources. Except Naga King, Tabasco and Punjab Longi, all lines belong to the species *C. annuum*. Naga King, popularly grown in Eastern India is believed to be a naturally occurring hybrid between *C. chinense* and *C. frutescens*; and Tabasco belongs to *C. frutescens*. Phylogeny of Punjab Longi is not clear but resembles that of *C. frutescens*. For diversity analysis, the germplasm was screened using 50 SSR markers of which 27 showed polymorphism (Table 1). Some of the markers used have been published (Lee et al., 2004) while others developed by AVRDC-The World Vegetable Center, Taiwan are unpublished.

Genomic DNA extraction and SSR analysis

The genomic DNA was extracted from fresh leaf tissues following CTAB method (Saghai- Maroof et al., 1984). Quality and quantity of DNA was checked both by gel electrophoresis and spectrophotometer. *In vitro* amplification was performed in a 96 well Eppendorf Master Cycler™ (Saiki et al., 1988). The gels were visualized under UV light and photographed using photo documentation system (UV Transilluminator).

The SSR allele sizes were determined depending on the position of bands relative to the ladder (Fermantas Gene Ruler 1 KB DNA ladder). Total number of alleles was recorded for each SSR marker in all the 64 genotypes by assigning allele number as 1, 2, 3, 4 and so on. The allele amplified in a particular set having highest molecular weight was numbered as allele 1. The amplified alleles were recorded as 1 (band present) or 0 (band absent) in a binary matrix. The polymorphic information content (PIC) values for all the primers were estimated using the formula:

$$PIC = 1 - \sum_{j=1} P_{ij}^2$$

Where, P_{ij} is the frequency of j^{th} allele in the i^{th} primer and summation extends over 'n' patterns.

Genetic diversity and cluster analysis

The SSR marker amplification profile of 64 genotypes was used to estimate genetic similarity based on number of shared amplified

Table 1. Allele amplification and PIC values for the polymorphic SSR primers screened using 64 chilli pepper accessions.

Primer	Primer sequence (5' - 3')	Number of alleles amplified	PIC Value
CAMS 117	F ttgtggaggaaacaagcaaa R cctcagcccaggagacataa	2	0.44
CAMS 072	F cccgcgaaatcaaggtaat R aaagctattgctactgggttcg	3	0.62
CAMS 885	F aacgaaaaacaaacccaatca R ttgaaattgctgaaactctgaa	3	0.43
CAMS 492	F gttcaaacacttccccctca R tgtcatcgttggtcgttacc	2	0.49
CAMS 647	F cggattcggttgagtcgata R gtgctttggttcggtctttc	3	0.62
CAMS 194	F tcatggaaaattaacaacgcata R gggggttggagaagaaagtt	3	0.60
AVRDC PP 195	F cggtgtgctaaatagtgcca R aacacaaaatgggaggtggt	2	0.49
AVRDC PP 166	F gcacgaggcttcatgtca R gcagcactgatcgacaaact	3	0.60
AVRDC PP 167	F tcatcttacacggcttgctc R agctcctcaactgcctttta	3	0.65
AVRDC PP 154	F cttcctagccacacacctca R gagcccaaaattcaaccagt	3	0.65
AVRDC PP 208	F cccctatctctttgctgctt R agctggggttttacaaatgg	3	0.63
AVRDC PP 65	F gtgaggccgagaatgaagat R aacgaccatgtgtggttga	3	0.65
AVRDC PP 67	F tattccttcttcacccctcc R gaaagaggcgctaactggac	3	0.58
AVRDC PP 3	F ctcgatgacttgatcgtga R cttgcattgtgaggtcactg	3	0.66
AVRDC PP 17	F ctactaccgctcctgctcct R agcttctgcttttggttcgt	3	0.64
AVRDC PP 18	F gctaggcttgatccttcacc R cgcttgaaatcatgctcact	3	0.78
AVRDC PP 24	F aaagcatgaaatcaccctcc R cggcaagaagatgaaagtca	3	0.66
AVRDC PP 32	F atggaggattacctcgcaac R catgatgaccatccatccat	4	0.69
AVRDC PP 205	F aacccccttcaaacttgttgc R gggggttcgaagtagatgaa	3	0.63
AVRDC PP 157	F gaattagctgcaacccaaca R gatttgtgatgccaccagac	3	0.64
AVRDC PP 174	F tcgttgttgggtggtacttg R ggaagatctcaaatgggtcg	2	0.50

Table 1. Contd.

AVRDC PP 187	F atcgtcgtcatccccatatt R aagataccatgccccttctg	3	0.39
AVRDC PP 188	F ctgctcttgaaaccgttgaa R cctctccatgaccctcctta	2	0.50
AVRDC PP 227	F attgattctgttggtggggt R ttccgtgtatcgctgctaac	2	0.48
AVRDC PP 239	F caaatgctgccactcacttt R acaacaaggggtgtttcctc	3	0.66
CAMS 679	F tttgcatgttttacccattcc R ccccaaaaattttccctcat	3	0.66
CAMS 806	F ggaccgttcaggaggttaca R gccatcattcaaaaccgaat	3	0.61
Total Average		75 2.78	- 0.59

Monomorphic primers were not included for estimating total and average value.

bands. The presence or absence of a particular amplification product was used as an index of genetic diversity/ relatedness using computer software package Windostat version 8.6. Clustering was done by UPGMA using SHAN module of Windostat version 8.6.

RESULTS AND DISCUSSION

Out of 50 primers screened, 23 primers did not show polymorphism and were not considered for further analysis. Twenty seven (27) primers were thus used for genetic diversity analysis on the basis of scoreable amplified bands. The number of bands amplified by each of the 27 primers ranged from two to four on superfine 2.5% agarose gel. A total of 75 alleles with an average of 2.78 alleles per locus were amplified in 64 genotypes. Maximum of four alleles were observed for primer AVRDC PP 32 and majority of the primers (20) amplified three alleles each (Table 1). The remaining six primers amplified two alleles each.

The PIC values provide an estimate of discriminating power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of these alleles. These values depend upon the genetic diversity among the accessions. Lower PIC values might be the result of closely related genotypes and the *vice-versa*. Senior et al. (1998) opined that marker loci with an average number of alleles running at equal frequencies will have the highest PIC value. The PIC values obtained in the present study varied from 0.39 for AVRDC PP 187 to 0.78 for AVRDC PP 18, with an average PIC value for 27 polymorphic primers to be 0.59. Based on the PIC values, it was found that primers AVRDC PP 18 was the most informative (0.78), followed by primers AVRDC PP 32 (0.69) and AVRDC PP 03 (0.66) whereas AVRDC PP 187, CAMS 117 and CAMS 885 with PIC values 0.39, 0.44 and 0.43, respectively, were the least informative. Our results were similar to those of Kwon et al. (2005) (0.53) and Yumnam et al. (2012) (0.52).

The SSR analysis revealed that the polymorphic level in this research was fairly high (54%) compared to some earlier reports indicating higher levels of genetic diversity among Indian accessions. For example, Paran et al. (1998) using 10 primer pairs detected 13% polymorphism in 34 Israeli gene bank accessions; Akatas et al. (2009) using four primer pairs found 26% polymorphism in Turkish germplasm; Kochieva and Ryzhova (2003) using nine primer pairs found 16.5% polymorphism in 14 Russian breeding lines; and Tam et al. (2005) using nine primer pairs observed 8.03% polymorphism. However, other workers using RAPD and AFLP techniques reported comparatively higher level of polymorphism. Oyama et al. (2006) screened the wild and domesticated pepper (*C. annuum* L.) populations of North-western Mexico where wild populations of *C. annuum* L. are widely distributed. Using RAPD markers, they reported higher levels of polymorphism in wild (all 166 band polymorphic) and domesticated (125 of 126 band polymorphic) populations. Geleta et al. (2005) obtained 352 polymorphic markers in the analysis of 39 accessions using six AFLP primer pairs. Using only four AFLP markers, Aktas et al. (2009) observed 215 bands, fifty-six (26%) of them were polymorphic indicating that AFLP markers are more efficient than the other marker systems. The varying levels of polymorphism in chilli pepper reported by various research groups could be attributed to the differences in genetic structures of the populations screened and the molecular techniques used. Samples collected from natural habitats of Mexico were expected to reveal higher levels of variability.

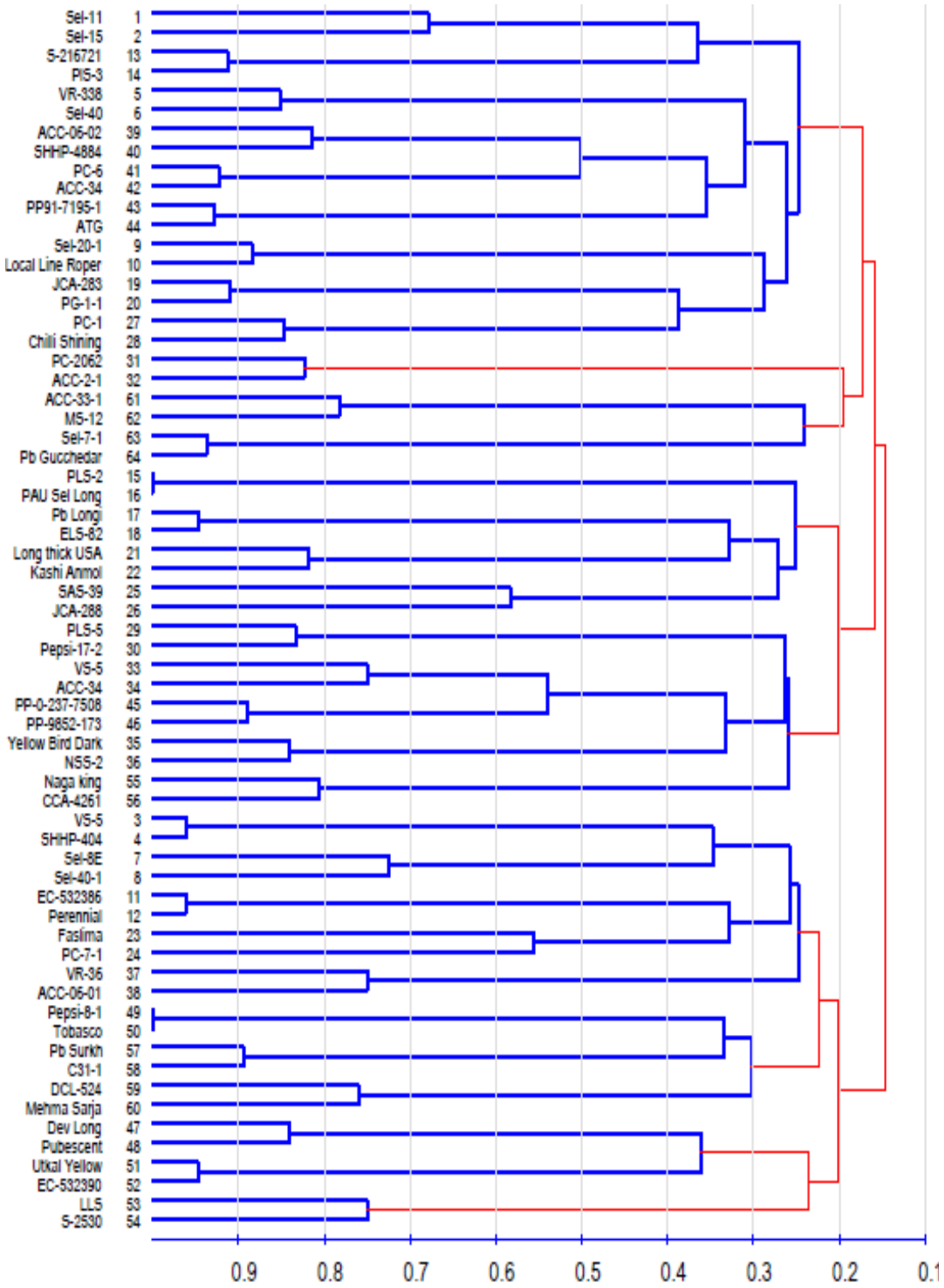

Figure 1. UPGMA based dendrogram of 64 chilli pepper genotypes based on 27 polymorphic SSR markers.

The diversity coefficient between any two genotypes estimated based on DNA amplification by SSR primers varied from 0.00 (between Pepsi 8-1 and Tabasco; and PLS-2 and PAU Selection Long) to 1.00 (between PC 2062 and Punjab Longi). The dendrogram showing genetic relationships among 64 genotypes based on SSR markers is presented in Figure 1. The UPGMA cluster analysis showed that all the 64 pepper genotypes were

clustered in to two main groups at similarity coefficient of 0.85. The larger group comprised of 42 genotypes and the smaller group comprised of 22 genotypes. Finally, the 64 genotypes were divided into nine sub- groups at similarity co-efficient of 0.75. The largest sub-group (Cluster 1) comprised of 18 genotypes and the smallest sub-groups (clusters 2 and 9) comprised of two genotypes each. The molecular analysis revealed that 222 pairs of genotypes have diversity coefficient of 0.90 or more, whereas only 12 pairs of genotypes have diversity coefficient of 0.10 or less.

Based on the molecular analysis, it was possible to characterize most of the accessions studied. However, the molecular analysis could not differentiate PLS 2 from PAU Sel Long; and Pepsi 8-1 from Tabasco. The probable reasons could be either due to the duplicates in the germplasm under different names or the present set of markers is not sufficient to detect differences between the two pairs of genotypes or due to the technological limitations. Though, the qualitative characters are subjective and hard to score; and the quantitative characters are influenced by the environment, yet it is unlikely that the two pairs of genotypes with contrasting morphological features are duplicates. Tabasco bears small erect fruits where as Pepsi 8-1 has medium long pendent fruits. Similarly, PLS 2 have broad leaves; and comparatively larger and pungent fruits, whereas PAU Sel Long have small leaves; and medium sized and mildly pungent fruits (Yadav, 2013). Molecular analysis with additional SSR markers and with greater genome coverage could help to reveal genetic diversity accurately and help to unambiguously differentiate those accessions with identical allelic patterns as revealed by the current set of SSR markers. The markers developed specifically for *C. annuum* and *C. frutescens* might give better results to differentiate Pepsi 8-1 from Tabasco, as the SSR markers some times are species specific. Another possible approach could be to go for AFLP markers which can reveal high frequency of DNA polymorphism even within cultivars as the genome coverage of these markers can be very high compare to SSR markers (Paran et al., 1998; Aktas et al., 2009; Kochieva and Ryzhova, 2003; Tam et al., 2005).

In the past, SSR markers have been used in successful prediction of heterosis and performance of F_1 hybrids from morphological similarity of their parents (Geleta et al., 2004). Due to facultative breeding nature (Tanksley, 1984) and availability of male sterility, it is easier to develop hybrids in chilli pepper. The current list of germplasm included accessions possessing both nuclear (NMS) and cytoplasmic (CMS) male sterility systems. Following this technique, parents have been identified for heterosis breeding. The pollen parents namely Pepsi 17-2, Acc 34, Utkal Yellow, EC 532390, Punjab Surkh, C 31-1, NSS 2 and VR 36 were found to be divergent from the CMS line PP-91-7195, with diversity coefficient of 0.90 or more. Similarly, Sel 7-1 and Punjab Guchhedar were found to be divergent from the CMS line PP 9852- 17; Punjab Guchhedar from PP 0-237-7508; Sel 11, LLS and DCL 524 from CCA 4261; PLS 2 and PAU Sel Long from PP-0-237-7; and ACC 06-01, Dev Long, Pubescent, Pepsi 8-1 and Tabasco from the NMS line MS 12. Due to their genetic divergence, the identified parents are expected to produce heterotic hybrids. Based on diversity analysis, parents Punjab Longi and PC-2062; Acc-0601 and Long Thick USA; Acc-0601 and Kashi Anmol; Yellow Bird Dark and LLS, PP 91-7195-1 and C 31-1; and ATG and C 31-1 with diversity coefficient of 0.97 or above were identified to attempt crosses and develop populations for mapping of useful genes, estimation of gene effects and to breed superior performing crop cultivars.

Conclusion

Our results based on the SSR analyses have vindicated that India is an important source of genetic variability of cultivated peppers, especially of hot pepper. The current set of primers is adequate to differentiate and characterize most of the chilli pepper genotypes studied. Based on the diversity analysis, parents have been identified for developing mapping populations, crop improvement and heterosis breeding.

Conflict of Interests

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

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