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Molecular analysis of soybean varying in water use efficiency using SSRs markers

Mithlesh Kumar[1,2]*and S.K. Lal[1]

[1]Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi-110 012, India
[2]Department of Genetics and Plant Breeding, C.P. College of Agriculture, S. D. Agricultural University, S.K. Nagar-385 506, India
*Corresponding Author's E-mail: mithleshgenetix@gmail.com

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Abstract

A set of 91 soybean germplasm lines, collected from different parts of the world, were screened for Water Use Efficiency (WUE) using Carbon Isotope Discrimination (CID) technique and were characterized for 10 quantitative traits. After screening under field condition, 44 soybean genotypes showed variations in WUE. Molecular diversity of these 44 diverse soybean lines was carried out with 26 Simple Sequence Repeats (SSRs) markers, of which 10 were polymorphic (38.47% polymorphism). 28 alleles were observed which were distributed over 10 loci, with an average of 2.8 alleles per locus. Polymorphism Information Content (PIC) value of 10 polymorphic markers ranged from 0.40 (locus Satt460) to 0.67 (locus satt260), with an average of 0.46. Pair-wise genetic similarity value, as calculated by simple matching coefficient, ranged from 0.99 to 0.40, with an average of 0.70. Genotypes were clustered using NTSYS-pc software employing unweighted paired group method using arithmetic averages to generate the dendrogram. Dendrogram exhibited 8 distinct clusters with a similarity coefficient of 0.69. Genotypes having low to medium and medium to high CID value were clustered in distant groups indicating usefulness of these polymorphic SSRs markers for differentiating genotypes on the basis of their CID value. The findings of this study indicate the need for broadening genetic base of the present Indian soybean cultivars through use of exotic sources of variation towards WUE. Thus, diverse genotypes identified in this study would be beneficial to soybean breeders to develop mapping population to identify QTLs for WUE.

Key words

Carbon isotope discrimination, Dendrogram, Molecular diversity, Polymorphism information content
Simple matching coefficient

Introduction

Soybean [*Glycine max* (L.) Merr.] is an annual herb belonging to family Fabaceae and subfamily Faboideae. It has been recognized as one of the premier agricultural crops in the world including India. Soybean plays an important role in supplying protein and oil. In addition, biodiesel produced from soybean oil reduces greenhouse gas emission and is beneficial for agriculture (Agarwal, 2007; Shi and Cai, 2010) as fuel. Insufficient water supply, especially during plant emergence, flowering and pod-filling stages leads to reduced yield of soybean (Abayomi, 2008). India is the leading soybean producing nation with an area of more than 9 M ha and ranks first in terms of production but 90% of soybean in India is cultivated as rainfed crop. Although genetic yield potential of currently available varieties is 2.5-3.0 tha^{-1}, the average national productivity is only 1.0 tha^{-1} (Lal and Rana, 2000).

Soybean growing season in India spreads over four months; it usually starts with the onset of monsoon in the second fortnight of June and ends in the month of September. However, the period might extend up to October depending upon the extent of delay in sowing to late arrival of monsoon. The rainfall and evapotranspiration analysis in India shows that there is recurrent drought during the month of September which affects grain formation resulting in low yield (Lal *et al.,* 2009). There is ample

rainfall during the month of July and August. However, the crop has to survive on residual moisture during the month of September. Under such conditions breeding for increased Water Use Efficiency (WUE) has been shown to be the most potent strategy for increasing productivity (Condon *et al.,* 2004). Measuring WUE is highly tedious and almost impossible, particularly, in large breeding population. Development of Carbon Isotope Discrimination (CID) technique (Farquhar *et al.,* 1982) is an alternative for measuring WUE in C3 species, which has made it possible to measure WUE in a large breeding population.

The basic requirement for any crop improvement programme is genetic variation for the trait of interest. Narrow genetic base due to its highly self pollinated nature is one of the major constraints in realization of full yield potential of soybean genotypes (Min *et al*., 2010). Assessment of the extent of genetic variability is fundamental for breeding, germplasm conservation and future hybridization programme. Earlier, only morphological traits were used for assessing genetic diversities. However, morphological characterization alone does not reliably portray genetic diversity and relationships among genotypes due to environmental interactions and unknown genetic control (Reif *et al*., 2005). On the other hand, molecular markers being DNA-based and independent from the environment assess genetic diversity in a more precise way. A number of molecular approaches are now available for such analyses, which have been proved to be genetically informative and useful for genotype identification and genetic diversity assessment (Wang *et al*., 2006; Nichols *et al*., 2007). Among the molecular markers, Simple Sequence Repeat (SSR) or microsatellite markers are being used widely for molecular characterization (Yoon *et al*., 2009; Guo *et al*., 2010). SSR markers are PCR-based, co-dominant, robust, reliable and are highly reproducible, with greater discriminatory ability than RFLP and RAPD markers. Use of CID technique for QTLs analysis to improve WUE in soybean has been reported earlier (Specht *et al*., 2001). However, the present study was conducted to screen a set of soybean genotypes for WUE using CID technique and to assess genetic diversity among genotypes using SSRs molecular markers to identify suitable genotypes for genetic improvement of soybean.

Materials and Methods

Plant material : Ninety one soybean genotypes comprising of released varieties, advanced breeding lines, exotic and indigenous collections were grown in the experimental field of Division of Genetics, IARI, New Delhi following augmented design (Federer, 1956) using 6 checks (PS 416, PS1024, DS 9814, SL444, EC472183 and DS 9712) in a 5 m single-row plot. Agronomic practices recommended for soybean were followed to raise a good crop. Data were recorded on the traits namely, Specific Leaf Area (SLA), leaf ash content, days to 50% flowering, days to maturity, pods per plant, branches per plant, seeds per pod, seed yield per plant and plant height. For assessing WUE, CID method, as suggested by Farquhar *et al*. (1989), was followed. Leaf samples of 60 day-old-plants under well watered conditions were collected from 10 randomly tagged plants in each genotype. Leaf samples from 5 plants were bulked together to make two replications for each genotype. Leaf samples were dried in hot-air oven at 80 ºC for 72 hrs and were ground to fine powder using mortar and pestle. One mg of dried leaf samples were combusted in the Flash elemental analyzer (NA 1112, Carlo Erba, Italy) interfaced to an Isotope Ratio Mass Spectrometer (IRMS; Delta-Plus, Thermo-Finnegan, Bremen, Germany) *via* continuous flow device (Conflo-III). Carbon isotopic composition of plant samples (d13Cp) was determined with analytical precision of less than 0.1%. Carbon Isotope Discrimination (D13C) was computed assuming isotopic composition of atmospheric air (d13Ca) to be 8% (Farquhar *et al*., 1989).

$$D13C\ (‰) = [d13Ca - d13Cp]/ [1 + d13Cp/1000].$$

All stable isotope measurements were made at the National Facility for Stable Isotope Studies, Department of Crop Physiology, UAS, Bangalore.

Molecular analysis : Fresh leaf samples (2.0 g) of soybean genotypes were collected from five young and healthy plants and frozen in liquid nitrogen (-196 ºC) followed by crushing to a fine powder using mortar and pestle. Genomic DNA from a pooled sample of 5 plants was isolated using CTAB method (Saghai-Maroof *et al*., 1984) with minor modifications. Purity of isolated DNA was checked in 0.8% agarose gel electrophoresis and were quantified using Spectrophotometer by taking readings at 260nm and 280nm. A set of 26 SSRs primers, spanning across the soybean genome, were selected for the present study. The primer sequences and related information were obtained from Cregan *et al*. (1999) and http://www.soybase.org. The primers were synthesised locally (Sigma Alderich Bangalore). The PCR amplification cycle consisted of initial denaturation at 94ºC for 2 min followed by 39 cycles consisting of denaturation, primer annealing and extension at 94ºC, 45º-55ºC and 72ºC, respectively for 1 min each followed by; the final extension step at 72ºC for 7 min. PCR was carried out in a volume of 20µl containing 50 ng of template DNA, 1 µM of each forward and reverse primers, 1.5mM dNTPs, 1 U *Taq* polymerase and 1X of buffer with $MgCl_2$ in Applied Biosystem Thermocycler (Gene AmpR PCR system 9700). Amplified products were resolved on 3% metaphor agarose gel along with 100 bp ladder (Bangalore Genei). Gel was run in 1XTBE buffer at a constant voltage of 70 V for about 4 hrs (until the tracking dye migrated to the end of the gel). Gel images were documented using gel documentation system (AlphaImager® HP). Amplified fragments displaying distinct sizes were considered to be different alleles.

Marker data analysis : Scoring of SSR alleles was performed manually in terms of positions of the bands relative to ladder sequentially from the smallest to largest. Absence of bands/

diffused bands/bands revealing ambiguity in scoring were considered as missing data and designated as '0' in comparison to '1' for the presence of alleles. Polymorphism Information Content (PIC) was determined by the following formula (Senior and Heun,(1993):

$PIC_i = 1 - \sum P_{ij}^2$; Where, P_{ij} is the frequency of j^{th} allele at i^{th} locus summed across all alleles in the locus.

Genetic similarity based cluster analysis : Simple matching coefficient (Sokal and Michner, 1958) was used as a measure of genetic similarity. Genetic similarity matrix (denoted as GS) was used to produce an agglomerative hierarchical classification by employing Unweighted Paired Group Method using Arithmetic Averages (UPGMA) which was used to assess the pattern of molecular diversity among 44 genotypes. All calculations were performed using NTSYS-pc (version 2.2) software (Rohlf, 2005). Bootstrapping was done using WINBOOT software (Yap and Nelson, 1996) with 5,000 replications.

Results and Discussion

44 soybean genotypes showed high, medium and low water use efficiency after being screened under field condition, using CID technique (Table 1). CID values were negatively associated with the trait of WUE (Kumar *et al*., 2012) *i.e*., genotypes with lower CID values were more water use efficient than genotypes with higher CID values. In the present study, genotypes were classified to have high WUE (CID less than 20.50 ‰), medium WUE (CID between 20.60 and 22.40 ‰) and low WUE (CID above 22.50 ‰) (Kumar *et al*., 2012). Among 44 genotypes, 5 genotypes *viz.,* EC471999, UPSL340-A, EC472137, EC472143 and EC472227 exhibited high WUE, 7 genotypes *viz*., EC389179, V9, UPSL309, PS1374, UPSL244, EC457323 and EC471292 exhibited low WUE and 32 genotypes *viz*., EC547191, DS9813, JS(SH)98-22, DS9719, UPSL332, PK1197, EC472129, HIS01, PS1024, M1094, EC457321, PK1135, EC471292, DS9814, PS1024, DS9801, PK1251, L440, SL459, and EC471998 etc. showed medium WUE (Table 1).

A total of 26 SSR primer pairs, distributed among 15 linkage groups of soybean were used to amplify specific SSR loci from each of the 44 selected genotypes showed moderate polymorphism. However, distribution of polymorphism was not uniform across the genome, some of the linkage groups (LGs) like L, C2 and O were found to be 100% polymorphic whereas K, Dlb, H, A1 ,E, F were 50 % and I was 25% polymorphic. Among these SSR primer pairs, 10 primer pairs produced clear single locus polymorphic bands (Table 2) indicating 38.47% polymorphism, which is moderate and these were used for further analysis. A total of 28 alleles from 10 SSR markers were detected across all the genotypes. The number of alleles per locus ranged from 2 to 3 with an average of 2.8. Moderate variations were observed with respect to CID value among the cultivated as well as germplasm accessions that can be exploited in soybean improvement programme. However, genetic variation among the genotypes was low at molecular level. The number of alleles identified using 26 SSR markers was markedly low. Li *et al*. (2008) studied genetic diversity of 1863 Chinese soybean landraces with 59 SSR loci and found an average of 19.7 alleles per locus. Guan *et al*. (2010) and Liu *et al*. (2011) reported an average of 16.2 and 7.14 alleles per locus, respectively. Incorporation of germplasm accessions and landraces in their studies has primarily

Table 1 : Carbon Isotope Discrimination (CID) value of 44 soybean genotypes used for molecular diversity analysis

Genotype	Source	CID Value
EC471999	Taiwan	19.95
UPSL340-A	Pantnagar	20.10
EC472137	Taiwan	20.24
EC472143	Taiwan	20.40
EC472227	Taiwan	20.49
EC547191	USA	20.58
UPSV24	Pantnagar	20.61
DS9813	Delhi	20.74
JS(SH)98-22	Sehore	20.79
DS9719	Delhi	20.80
UPSL332	Pantnagar	21.12
PK1197	Pantnagar	21.18
UPSL303	Pantnagar	21.18
EC472129	Taiwan	21.23
HIS01	India	21.25
PS1024	Pantnagar	21.26
M1094	India	21.41
PS416	Pantnagar	21.48
EC457321	USA	21.50
EC457285	USA	21.55
EC456646	USA	21.67
PK1135	Pantnagar	21.72
EC471292	Taiwan	21.81
EC457196	USA	21.84
DS9814	Delhi	21.88
PS1042	Pantnagar	21.94
DS9801	Delhi	22.10
DS9816	Delhi	22.10
PK1251	Pantnagar	22.10
EC472130	Taiwan	22.13
DS9819	Delhi	22.14
EC471784	Taiwan	22.18
L440	Taiwan	22.19
SL459	Ludhiana	22.25
EC457505	USA	22.29
EC471923	Taiwan	22.33
EC471998	Taiwan	22.33
EC389179	Taiwan	22.42
V9	Almora	22.53
UPSL309	Pantnagar	22.69
PS1374	Pantnagar	22.77
UPSL244	Pantnagar	22.80
EC457323	USA	22.82
EC471292	Taiwan	22.92

Table 2 : List of simple sequence repeat markers found polymorphic during molecular characterization of soybean genotypes

SSR markers	Linkage group (LG)	cM Position on LG in soybean genome map	Motif	Sequences of SSR Markers(5'-3')	Number of alleles	PIC* value
Satt157	Dlb	37.07	$(AAT)_{31}$	F: GGGCTCACTCTCGATAGTAGGTATAAAG R: GGGATACCAAAAGGAATAATTGTCTT	3	0.46
Satt434	H	105.74	$(ATA)_{32}$	F: GCGTTCCGATATACTATATAATCCTAAT R: GCGGGGTTAGTCTTTTTATTTAACTTAA	3	0.42
Satt545	A1	71.39	$(TTA)_{24}$	F: CAATGCCATTCCATATTTGTT R: CAATTGCCCTAGTTTTGATAG	3	0.50
Satt664	L	92.66	$(ATA)_{10}$	F:GCGTAGATGCTCAACATCAACACTAATCTG R: GCGGACGATGAAGAAATATACTATTACGAA	3	0.46
Satt685	E	56.70	$(AAT)_{14}$	F: ATCGTGGCATGTCTCACTAC R: GAGGCGGAAGGAAATCTAAT	2	0.42
Satt260	K	80.12	$(ATT)_{22}$	F: GCGCCAAATGTATACTTTAAATTCTT R: GCGGGTTTAGCTAAAATAGTTCGTGC	3	0.67
Satt330	I	77.84	$(ATT)_{18}$	F: GCGCCTCCATTCCACAACAAATA R: GCGGCATCCGTTTCTAAGATAGTTA	3	0.59
Satt331	O	93.37	$(ATT)_{14}$	F: GCAGAGTCCCCCCTAAATATAG R: CGGGAACAACCACACTCTCCATT	2	0.63
Satt460	C2	117.77	$(ATT)_{26}$	F: GCGCGATGGGCTGTTGGTTTTTAT R: GCGCATACGATTTGGCATTTTTCTATTG	3	0.40
Satt522	F	119.19	$(ATT)_{16}$	F: GCGAAACTGCCTAGGTTAAAA R: TTAGGCGAAATCAACAAT	3	0.65

F = Forward Primer; R = Reverse Primer; *PIC=Polymorphism Information Content

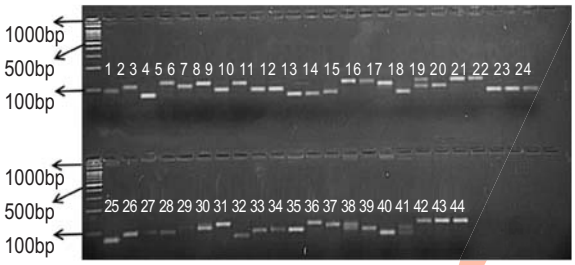

Fig. 1 : Diversity among the 44 soybean genotypes amplified with primer set Satt522 along with 100bp ladder. [Lane sequences in the gel corresponds to the identification of the genotypes as indicated in Table 1]

contributed towards detection of more number of alleles per locus. The plant material in the present study consisted of 24 Indian released cultivars and 20 exotic germplasm collected from other parts of the world. The present study is in accordance with those of Burnham *et al*. (2002) who detected less allelic richness with average of 2.59 alleles per locus in his study of 88 soybean genotypes (included cultivars and accession) screened for *Phytophthora* resistant. Kumar *et al*. (2014) also reported an average of 2.36 alleles per locus among the diversity panel of 96 soybean genotypes which was close to the findings of Liu *et al*. (2011) who detected an average of 6.55 alleles per locus. Among the 10 polymorphic markers, Satt260, Satt522, Satt157, Satt460, Satt664, Satt434, Satt330 and Satt545 had three alleles per locus and other two markers like Satt685 and Satt331 had two alleles per locus. Fig.1 shows an example of DNA profile at Satt522 locus having 3 distinct alleles among the soybean genotypes. About 12.5% of the alleles occurred at a frequency of 0.25 or less, 41.65 % at 0.25-0.50 and 48.4% at frequency more than 0.50; which

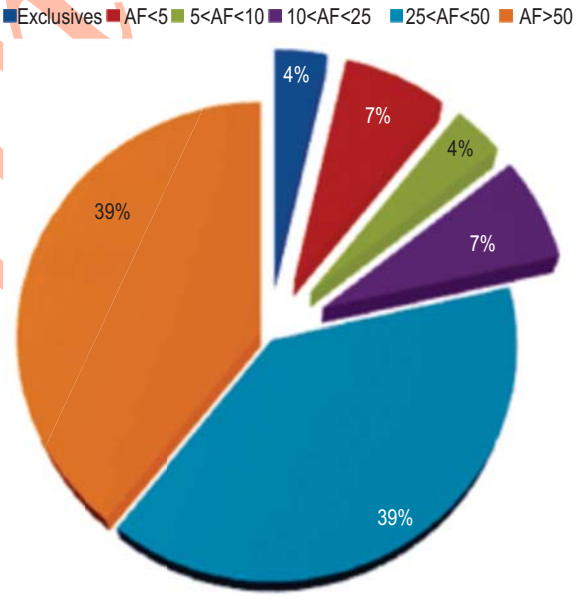

Fig. 2 : Pie-diagram showing allelic frequencies and allele distribution across 44 soybean genotypes [AF= Allele frequency]

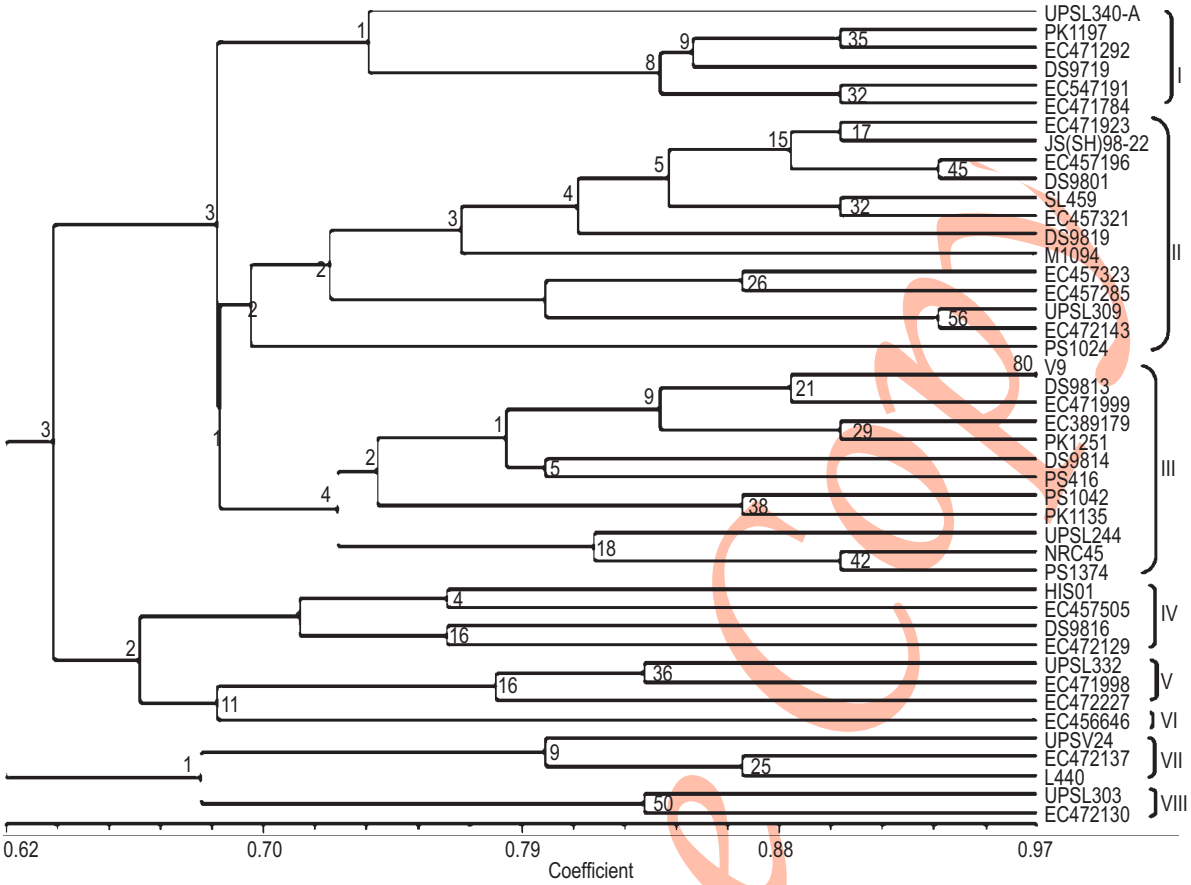

Fig. 3 : Dendrogram of 44 soybean genotypes derived using UPGMA cluster analysis based on simple matching coefficient [values at nodes indicate bootstrapping data]

indicates less genetic variability among these genotypes (Fig.2). Unique allele specific for UPSL340 were observed in the present study, which may be useful for DNA fingerprinting. PIC values of 10 polymorphic SSR primers ranged from 0.40 at locus Satt460 to 0.67 at locus satt260 with an average of 0.46 (Table 2).

Similarity coefficient was found to be highest between DS9813 and V9 genotypes (0.99) followed by PK1135 and EC472143 (0.94), EC457196 and EC457321 (0.94). Twelve pairs of genotypes showed very high value of similarity coefficients (0.90). Lowest similarity coefficient was observed between four pairs of genotypes namely, PK1197 and EC472130 (0.40); HIS01 and UPSL303 (0.40); EC472130 and EC457505; and NRC45 and EC47227 (0.40). Genotypes EC457196 exhibited highest average similarity (0.75) with all the genotypes and EC472130 exhibited lowest average similarity (0.60) with all the genotypes.

UPGMA cluster analysis, based on Genetic Similarity (GS) matrix, resulted in eight groups that can be distinguished by truncating the dendrogram at the cut-off value of 0.69 (Fig.3). Number of genotypes varied among different clusters in the dendrogram. Maximum number of genotypes was observed in cluster II (13 genotypes) followed by cluster III (12 genotypes); while the cluster VI represented single genotype. CID values of the genotypes were classified as low, medium and high among the eight clusters (Table 1). Cluster I contain genotypes having low to medium CID value except EC471784 having high CID value. Clusters IV, V and VII were distant from cluster I and contained genotypes having medium to high CID value except EC472227, which showed low CID value. These findings showed that the polymorphic SSR primers observed in the present study were useful to distinguish genotypes for WUE or CID value.

The present study established that Indian soybean cultivars have narrow genetic base and moderate variability with respect to WUE. The present study also confirmed the power of SSRs in characterizing and developing DNA fingerprints of soybean cultivars. The identified genotypes will be good base

materials for developing water use efficient cultivars in India.

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