Identification of unique alleles and assessment of genetic diversity of soybean genotypes using SSR markers and seed traits

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Abstract
Thirty polymorphic SSR markers and seven seed traits were used to determine the genetic diversity among twenty five soybean genotypes that differ with respect to seed coat color. A total of 133 alleles were detected and the number of alleles for each SSR locus varied from two to seven with an average of 4.0. Polymorphic information content varied from 0.81 to 0.07. Jaccard’s similarity coefficient grouped the genotypes into two major clusters. To test the goodness of fit of clusters to SSR markers, cophenetic correlation was estimated. Cophenetic value of 0.98 indicated a very good fit. A combination of SSR and seed traits data grouped the genotypes into two major clusters with grouping of all the brown seeded genotypes into one subcluster. A total of 21 unique alleles were identified in twenty five soybean genotypes which is a valuable resource for DNA fingerprinting studies. A set of nine SSR markers (Satt 600, Satt 463, Satt 371, Satt 193, Satt 538, Satt 126, Satt 286, Satt 281 and Satt 656) could differentiate all the soybean genotypes.

Key Words
Genetic diversity, SSR markers, Soybean, unique alleles, seed traits

INTRODUCTION
Soybean (Glycine max L. Merrill) is an important legume crop grown for protein and oil. The high nutritional value of 40 per cent protein and 20 per cent oil makes it a miracle crop of the twentieth century. Around 55 per cent of total vegetable oil in the world is contributed by soybean crop. India ranks fifth in soybean production (10.98 m.t.) in the world with area of 10.47 m.ha and productivity of 1049 kg/ha. A prerequisite for crop improvement is the study of existing genetic variability in the germplasm for utilization in breeding program. Studying genetic variability of soybean germplasm differing for seed coat colour helps in the selection of parents for development of mapping populations and to derive transgressive segregants with respect to seed longevity and widening the genetic base (Kumawat et al., 2015). Cultivars suitable for different agro climatic conditions were developed by soybean researchers through introduction, selection, mutation, hybridization of elite cultivars and germplasm followed by systematic breeding and evaluation programmes (Chauhan et al., 2015). The use of DNA markers for germplasm characterization in addition to morphological traits provides additional information which is highly useful for germplasm registration and protection. SSR markers have been widely used in the genetic diversity
Identification of unique alleles and assessment studies of the soybean germplasm collections worldwide and high levels of polymorphism at SSR loci have been reported for both the number of alleles per locus and gene diversity (Wang et al., 2006a, 2010; Fu et al., 2007; Wang and Takahata, 2007; Li et al., 2008; Singh et al., 2010; Tantasawat et al., 2011). With advancement in science, DNA markers associated with specific traits are identified and utilized in germplasm characterization and marker assisted selection. The present investigation on molecular characterization of soybean germplasm has been carried out to assess the existing genetic diversity and identification of genotype specific markers.

MATERIALS AND METHODS

Twenty-five soybean genotypes having variable seed coat colour were collected from Agricultural Research Station, Adilabad, Prof. Jayashankar Telangana State Agricultural University (PJTSAU), Telangana (Table 1) as seed coat colour plays a prominent role in seed longevity. The germplasm in this study included released cultivars and advanced breeding lines. Seed traits such as seed coat colour, 100 seed weight, seed length, seed width, seed thickness, seedling length and seedling dry weight were recorded. The seed traits excluding seed coat colour were classified as small, medium, large and extra-large. Data was scored as 0 or 1 and analysed along with SSR data using NTSYS-pc ver.2.02.

Genomic DNA was isolated from young leaves following the CTAB (cetyl trimethyl ammonium bromide) procedure as described by Saghai-Maroof et al. (1984). Quantification was accomplished by analyzing the DNA on 0.8% agarose gel stained with ethidium bromide using diluted lambda DNA as standard. The genomic DNA was diluted to 50 ng/μl for PCR. A total of 34 SSR markers distributed across the integrated linkage map of soybean (Cregan et al., 1999) were used. The primer sequences of SSR markers along with the annealing temperatures are presented in Table 2. DNA was amplified in a total reaction volume of 10μl with the following cycling conditions: Initial denaturation at 94°C for 5 min. followed by denaturation at 94°C for 1 min. annealing at 48-58°C (based on primer Tm) for 30 sec. and extension at 72°C for 45 sec. This cycle was repeated 35 times, followed by 7 min. final extension at 72°C. The amplified products were separated on 3% MetaPhor agarose gel and detected by ethidium bromide staining. The gel was visualized in UV transilluminator and photographed using gel documentation system.

The PCR products were analyzed by scoring qualitatively for the presence (1) or absence (0). Polymorphism Information Content (PIC) was calculated according to Anderson et al. (1993) using the following equation:

\[ PIC_j = 1 - \sum_{i=1}^{n} P_i^2 \]

Where, \( i \) = the \( i \)th allele of the \( j \)th marker, \( n \) = the number of alleles at the \( j \)th marker and \( P \) = allele frequency.

The similarity matrix was analysed using NTSYS-pc ver.2.02 to produce an agglomerative hierarchical classification by employing UPGMA with average linkage. Genetic similarity matrices were generated based on polymorphic SSR markers and seed traits (NTSYS-pc2.02).

RESULTS AND DISCUSSION

Seed storability is the major constraint in soybean seed production. The present investigation deals with the study of genetic diversity among twenty five coloured soybean genotypes based on the seed traits and SSR markers.

Table 1. List of soybean genotypes used in the present study

| Genotype       | Seed Coat Colour | Genotype  | Seed Coat Colour |
|----------------|------------------|-----------|------------------|
| CAT 195 BR4    | Brown            | DS91-3    | Yellow           |
| CAT 192 BR15   | Brown            | EC 12503  | Yellow green     |
| CAT 1622 TG X 302-2A | Brown         | CAT 971-GP2 | Yellow green     |
| CAT 194 BR3    | Brown            | G828      | Yellow           |
| NG 1142 CHICO  | Brown            | NRC-130   | Yellow           |
| NRC 2755       | Brown            | DS 24110  | Yellow           |
| CAT 243 DE 201 | Black            | EC 113416 | Yellow green     |
| UPSL 387       | Black            | KARUNE    | Green            |
| KALITUR        | Black            | PSPB-23   | Green            |
| G1922          | Black            | CAT 2059 GC 84058-18-4 | Green |
| TG X 849D-13-4 | Black            | AGS 12 CAT25-A | Yellow  |
| IC 16572       | Black            | NRC 105   | Green            |
| CAT 1852 TG X 854-25D | Black      |           |                  |

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as seed coat colour is a predominant trait affecting seed storability. The information is highly useful for plant breeders in selection of parental lines for hybridization program aimed at improving seed storability in soybean.

Among all the available DNA markers, SSRs have been used successfully in estimation of genetic diversity and studying the relationships among soybean genotypes in different populations (Wang et al., 2006a,b; Guan et al., 2010; Wang et al., 2010; Jain et al., 2017). A total of thirty-four SSR markers distributed on 18 of 20 linkage groups of soybean were used for studying genetic diversity among which twenty five soybean genotypes having varying seed coat colour. Among 34 SSR markers studied, 30 were polymorphic and produced 133 alleles. The number of alleles for each SSR locus varied from two (Satt 598, Satt 162, Satt 619 and Satt 285) to seven (Satt 534 and Satt 133) with an average of 4.0 (Table 3). The size of the alleles ranged from 78 to 346 bp (Fig. 1). Polymorphic information content is a measure of the allelic differentiation. The highest PIC value was observed for the marker Satt 126 (0.81) and the lowest for Satt 285 (0.07) (Table 3). A combination of nine SSR markers (Satt 600, Satt 463, Satt 371, Satt 193, Satt 538, Satt 126, Satt 281 and Satt 656) was able to differentiate all the 25 soybean genotypes. In addition, 21 rare alleles (allele having frequency of less than 5%) from 30 SSR loci were identified. A maximum of two rare alleles/SSR locus were identified (Satt 463, Satt 565, Satt 631, Satt 686 and Satt 133). It was also observed that the frequency of rare alleles was much higher at SSR loci which have large number of alleles (Jain et al., 2004; Gupta and Manjaya, 2017).

Table 2. List of SSR primers used in the present study

| Primer name | Forward primer | Reverse primer | Annealing temp °C |
|-------------|----------------|----------------|------------------|
| Satt 286    | GCGGCCTTAATTATAGCTCCCGAAA | GCGTTTGCTAGAATGTCTTCTCA | 55               |
| Satt 534    | CTCTCTCTGCGCAACACAAATA | GGGGATCTAGGCGCATGAC | 60               |
| Satt 565    | GGGCCCGGGACTTGTAAACCTAAT | GGGCTCTCTATGATGTCATATAAA | 55               |
| Satt 371    | TGCAAACACTGCTAGTTACCTCA | GAGATCGCGAAATTTAGTGTAACCA | 55               |
| Satt 184    | GGGCTATGAGATATTCAAAATTGGCC | GGGCATCTGTCTTACTCAT | 51               |
| Satt 619    | GGGGAGACTAGCTGCTCTCTGATT | GGGCTTAGCATATAAGACTATGACTATCG | 53               |
| Satt 481    | GGGTAACTGCTCAACCACACTTAT | GGGCTGTAAACGTGGTGAAAGAAT | 48,50            |
| Satt 463    | TTGGATCTCATTACATCTCTTAACAG | CTGCAAATTTGATGCACATGTGCTA | 52               |
| Satt 193    | GGGTTTCTGATATGAAATATGTTACCTC | TGGTTCGCTATGGTACATGAAC | 48               |
| Satt 175    | GACCTGCCTCTGTTCTCCTCAT | GGGTGACCACCCCTATTCCTTAT | 53               |
| Satt 598    | CAGTTTGAATATTACCTACCTGCTATTAA | CACATAAATGGTGCCTGTATATTCT | 48,50            |
| Satt 281    | AAGCTTCCATACGCTGCTTCAAC | TGCATGGCGAGAAGAAGATG | 50               |
| Satt 389    | GGGCGTGTTGATGTTGGAATCTCA | GGGCCCAAACCAAGAAGATAC | 55               |
| Satt 538    | GGGCGCTATCATTCTCAAAGCAAG | GGGCCGAATAAATAGAAGAGGAAGAGA | 51               |
| Satt 600    | GGGCGAGAAAAAACGCTTATTTATTTTT | GGGCAATCGTCAGGTGTTTATT | 52               |
| Satt 285    | GGGCATGATTGGATTAAAAACATACCTT | GGGGATCTAGGCGCATGATG | 50               |
| Satt 434    | AGGTTTGGGTGAATACCTAAACTCTAAT | GGGGCGGATTGGTGTTTTGGTCAG | 52               |
| Satt 162    | GGGGCGGATTGGTGTTTTAGCAGATG | GGGTTAATATATTCTCTAATAGTTT | 48               |
| Satt 523    | GGGATTTCCTCTCCTGAAGAATTTCTG | GGGCTTTTCTGGTCCTTTATTTTAT | 53               |
| SOYGPATR    | GGAAGAAATGGTTGCTGTG | GGGAGAGAGGGTGGAGATT | 54               |
| Satt 631    | GGGTAGATCAGGAGGTGGTACG | GGGGATCTAGGCGCATGATG | 55               |
| Satt 126    | GCTGGTGATTGTTGGAAGAA | AAAAAAGAAAGTCAGGTGAT | 55               |
| Satt 129    | TTCAGTACAGGGAAGGTGATAAATAAAT | TGGATGTGGGACTTAAAGGAT | 55               |
| Satt 168    | CGCTGGCCCAAAAATTAATGTA | CCATTCCAAAACCTATAT | 56               |
| Satt 656    | GGGTGCTTCTTTCTTGTTTCTTGGT | GGCTGTCAGTAGTTGGATAGAAATG | 55               |
| Satt 686    | AGGGAAAATAAGGAAAAGTAGAGA | GGCTTATCGAAGAAGAGGAGAAG | 55               |
| Satt 289    | GGGCCAGGTTTTAAAAGT | CTGGCCATCATCTAGCCTTCTTCT | 55               |
| Satt 133    | GCAAATTGAAAGAAAAGATGGATT | TAAAAGGATGGTTGAAGAAGAAG | 56               |
| Satt 390    | AGGGTGCTGATTAAAATAGTAC | ATACCCGCGGACATAATTC | 55               |
| Satt 038    | GGGATCTTCTTTCTTTCTTATTAGGT | GGCGGATTGAAATGGTGGTATGCA | 55               |
Table 3. List of SSR primers for genotyping of 25 soybean genotypes

| SSR locus | Allele size range (bp) approximate | Number of alleles | PIC value | Rare alleles |
|-----------|-----------------------------------|-------------------|-----------|--------------|
| Satt 600  | 150-210                           | 4                 | 0.71      | 0            |
| Satt 463  | 110-200                           | 5                 | 0.65      | 2            |
| Satt 286  | 188-238                           | 4                 | 0.68      | 1            |
| Satt 371  | 250-278                           | 5                 | 0.75      | 0            |
| Satt 481  | 78-110                            | 4                 | 0.64      | 0            |
| Satt 281  | 190-240                           | 5                 | 0.75      | 1            |
| Satt 285  | 195-200                           | 2                 | 0.07      | 1            |
| Satt 619  | 112-125                           | 2                 | 0.43      | 0            |
| Satt 175  | 150-165                           | 4                 | 0.57      | 1            |
| Satt 523  | 110-135                           | 5                 | 0.72      | 0            |
| Satt 162  | 300-320                           | 2                 | 0.58      | 0            |
| Satt 193  | 215-250                           | 6                 | 0.80      | 0            |
| Satt 538  | 110-135                           | 4                 | 0.59      | 1            |
| Satt 038  | 164-200                           | 4                 | 0.56      | 0            |
| Satt 434  | 325-346                           | 6                 | 0.75      | 1            |
| Satt 598  | 165-175                           | 2                 | 0.14      | 0            |
| Satt 534  | 148-188                           | 7                 | 0.81      | 1            |
| Satt 565  | 158-200                           | 6                 | 0.70      | 2            |
| Satt 184  | 148-183                           | 5                 | 0.70      | 1            |
| Satt 129  | 117-150                           | 4                 | 0.60      | 1            |
| Satt 168  | 154-206                           | 4                 | 0.65      | 0            |
| Satt 631  | 116-172                           | 6                 | 0.76      | 2            |
| Satt 126  | 120-164                           | 6                 | 0.81      | 0            |
| Satt 656  | 135-153                           | 5                 | 0.75      | 0            |
| Satt 686  | 261-293                           | 4                 | 0.56      | 2            |
| Satt 289  | 202-217                           | 4                 | 0.67      | 1            |
| Satt 389  | 226-250                           | 4                 | 0.69      | 0            |
| Satt 133  | 213-293                           | 7                 | 0.80      | 2            |
| SOYGPATR  | 116-124                           | 4                 | 0.64      | 1            |
| Satt 390  | 243-252                           | 3                 | 0.55      | 0            |

Genetic similarity matrices of soybean genotypes were generated using polymorphic SSR markers. The genetic similarity coefficient between individuals based on SSR data ranged from 0.07 [CAT-2059-GC-84058-18-4 (green) and AGS12-CAT25A (yellow)] to 0.51 [UPSL-387 (black) and NRC-130 (yellow)]. The dendrogram based on UPGMA clustering clearly classified the twenty-five soybean genotypes into two distinct clusters indicating the diverse nature of the germplasm (Fig. 2). Cluster I divided into two subclusters IA comprising of three genotypes - CAT 195 BR4& NG1142 CHICO (brown), DS91-3 (yellow); IB having ten genotypes of which five are black seeded-IC 16572, G1922, CAT 243 DE201, TGX849D-13-4& UPSL 387 (black), CAT 971 GP2, AGS12 CAT25A& NRC 130 (yellow), EC 12503 (yellow green), KARUNE (green). Cluster I is further classified into two subclusters IIA & IIB. Cluster IIA having five genotypes [DS24110 (yellow), EC 113416 (yellow green), CAT 2059GC84058-18-4 (green), G828 (yellow), NRC 2755 (brown)] and IIB with seven genotypes [PSPB 23, NRC 105 (green), CAT 194 BR3, CAT 1622 TGX302-2A, CAT 192 BR15 (brown), KALITUR, CAT 1852 TGX854-25D (all black)]. To test the goodness of fit of clustering to a set of data cophenetic correlation or cophenetic value was estimated using the COPH and MXCOM options in NTSYS-pc2.02 program. The cophenetic value of 0.98 obtained using the SSR data indicated a very good fit of clustering to SSR data. Although thirty polymorphic SSR markers have been used, however as few as nine SSR markers (Satt 600, Satt 463, Satt 371, Satt 193, Satt 538, Satt 126, Satt 286, Satt 281 and Satt 656) could differentiate the 25 soybean genotypes.
Maintenance of seed longevity from harvest to till the subsequent sowing is the biggest challenge that needs to be addressed in soybean. In general, the brown and black seeded genotypes have good storability compared to yellow or green seeded types (Hosamani et al., 2013). Molecular diversity existing among the good and poor storer genotypes may be exploited by including them in crossing program for improving seed storability in soybean. Jaccard’s similarity coefficient of 0.22 between the good storer genotypes PSPB-23 (green seeded), CAT-1852-TGX854-25D (black seeded) and the poor storer NRC 130 (yellow) (based on accelerated ageing test) indicated diverse nature of these genotypes. Hence the molecular diversity existing among the good and poor storer genotypes may be exploited by including them in crossing program for improving seed storability in soybean.

Genetic diversity among the soybean genotypes using SSR markers was also assessed in combination with seed traits that determine seed longevity and seed vigour such as seed coat colour, 100 seed weight, seed length, seed width, seed thickness, seedling length and seedling dry weight. The genetic similarity coefficient ranged from 0.04 [TGX849D-13-4 (black) and NRC 2755 (brown)] to 0.57 [CAT-1622-TGX302-2A (brown) and CAT-194-BR3 (brown)]. Cluster analysis grouped the genotypes into two major clusters (Fig. 3). Cluster I is divided into two sub clusters (IA & IB) and all the brown seeded genotypes were grouped in IA indicating more similarity among the genotypes. Cluster II is the smallest with only two yellow seeded genotypes (KARUNE& NRC 105). Seed traits had dominant role in the clustering of the genotypes. Cluster II is the smallest with only two yellow seeded genotypes (KARUNE& NRC 105). Seed traits had dominant role in the clustering of the genotypes. Majority of the clusters had genotypes of single coat colour. Five clusters were common in both SSR and SSR + seed traits-based grouping. Several studies to assess genetic diversity based on yield/ yield contributing traits and seedling traits have been carried out to identify the promising parental lines for hybridization-based crop improvement program in soybean (Kachhadia et al., 2014; Manav and Arora, 2018).
Fig. 2. Phylogram of twenty five soybean genotypes based on Jaccard similarity coefficient of thirty polymorphic SSR loci.
Fig. 3. Dendrogram of twenty-five soybean genotypes based on Jaccard’s similarity coefficient of thirty polymorphic SSR data and seed traits.
Table 4. Unique alleles for soybean genotypes

| S.No. | Primer | Allele size (bp) approximate | Genotypes with unique allele |
|-------|--------|-------------------------------|----------------------------|
| 1     | Satt 463 | 135                           | NRC 2755                    |
| 2     | Satt 463 | 200                           | CAT2059 GC 84058-18-4       |
| 3     | Satt 286 | 188                           | PSPB-23                     |
| 4     | Satt 281 | 198                           | NRC 2755                    |
| 5     | Satt 285 | 195                           | KARUNE                      |
| 6     | Satt 175 | 165                           | NRC 105                     |
| 7     | Satt 538 | 110                           | PSPB-23                     |
| 8     | Satt 434 | 330                           | NG 1142 CHICO               |
| 9     | Satt 534 | 158                           | NRC 2755                    |
| 10    | Satt 565 | 163                           | NRC 2755                    |
| 11    | Satt 565 | 190                           | NG 1142 CHICO               |
| 12    | Satt 184 | 153                           | NRC 105                     |
| 13    | Satt 129 | 150                           | G828                        |
| 14    | Satt 631 | 165                           | NRC 2755                    |
| 15    | Satt 631 | 172                           | PSPB-23                     |
| 16    | Satt 686 | 261                           | DS91-3                      |
| 17    | Satt 686 | 271                           | AGS12 CAT 25A               |
| 18    | Satt 289 | 217                           | PSPB-23                     |
| 19    | Satt 133 | 276                           | NRC 2755                    |
| 20    | Satt 133 | 293                           | NRC 105                     |
| 21    | SOYGPATR | 124                           | NRC 2755                    |

SSR marker information pertaining to unique and rare alleles have a high discriminatory power and can serve as diagnostic markers for unambiguous soybean varietal identification/DNA fingerprinting (Gupta and Manjaya, 2017). A total of 21 unique alleles were amplified by sixteen SSR loci in twenty five soybean genotypes in a size range of 110-330bp (Satt 463, Satt 286, Satt 281, Satt 285, Satt 175, Satt 538, Satt 434, Satt 534, Satt 565, Satt 184, Satt 129, Satt 631, Satt 686, Satt 289, Satt 133 and SOYGPATR) (Table 4). The markers Satt 463, Satt 565, Satt 631, Satt 686 & Satt 133 amplified two specific amplicons each in seven different genotypes. SSRs have been shown to produce the highest polymorphism compared to other marker systems such as RFLPs, AFLPs and RAPDs, and much greater ability to identify unique alleles in elite and PI (plant introduction) soybean germplasm when compared to other marker systems (Narvel et al., 2000; Wang et al., 2006a). Earlier studies involving ninety soybean cultivars resulted in the identification of eight SSR markers for DNA fingerprinting. The study also revealed the presence of fifty four rare alleles including nineteen genotype specific or unique alleles (Gupta and Manjaya, 2017).

The study revealed diverse nature of soybean genotypes with respect to seed traits and SSR markers which may be utilized in soybean varietal improvement program to enhance seed longevity. The rare alleles/unique alleles of SSR markers are important genomic resources for soybean germplasm characterization/DNA fingerprinting.

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