Genetic Diversity as Assessed by Agronomical Traits and Molecular Markers in Local Land Races of Green gram (Vigna radiata L.) Under Rice Fallow Condition

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Abstract  Twenty five greengram genotypes were analysed for assessment of genetic diversity through study of agronomical traits and molecular markers. Multivariate analysis of divergence among 25 green gram genotypes for 12 agronomical traits revealed considerable genetic diversity and led to their grouping into four clusters. Reaction of 10 days old plants on exposure to cold contributed maximum, accounting 28.5% of total divergence. A set of 44 SSR markers derived from adzuki bean and greengram was used. Nineteen SSR markers showed amplification and out of which 17 showed polymorphism with an average of 1.63 polymorphic alleles per primer pair. The dendogram constructed based on SSR markers grouped the 25 greengram genotypes into six main clusters. The Jaccard’s similarity coefficient ranged from 0.42 to 0.94. The clustering pattern based on SSR polymorphic data showed consistency with that of multivariate analysis with little deviation.

Keywords  Agronomical traits; Genetic diversity; Greengram; Molecular marker; SSR

Background  Greengram (Vigna radiata L. Wilczek), also known as mungbean, with genomic size of 579 Mbp (Somta and Srinives, 2007) is an important pulse crop of India. It is cultivated in an area of 3.55 m ha with a productivity of 512 kg/ha. (Project Coordinator Report, 2011-12). A substantial part of Kharif rice area (11.65 m ha) remain fallow during rabi season in India. Of these 82% of the rice fallow are located in the states like Odisha, Assam, Bihar, Madhya Pradesh and Chhattisgarh in India (Subbarao et al., 2012), where green gram can be grown profitably on residual moisture with minimum irrigation. Due to lack of suitable high yielding varieties for rabi rice fallow situation, the greengram rice fallow area is diminishing day by day.

The narrow genetic base of present day greengram cultivars sharing some common parents in their pedigree is one of the major constraints in breeding programme for rice fallow condition. More than 70% total mungbean area of Odisha is under rice fallow. These areas of the state are covered by a large number of local greengram genotypes which are defeating to local agroclimatic situation with many desirable traits but having low productivity. They can be used in breeding programme for development of suitable varieties for rice fallow condition. For this purpose, the knowledge about the nature and magnitude of genetic divergence is essential for selection of diverse parents for hybridization programme to get a wide spectrum of gene recombination for quantitatively inherited traits.

Agronomical traits play a major role in studying and characterizing germplasm since long. Molecular marker analysis in conjunction with morphological and agronomic evaluation data is recommended to increase the resolving power of genetic diversity analysis and provide complimentary information (Shrivastav et al., 2012). In recent years, more sensitive DNA based techniques like SSR markers are developed which are more suitable because of easiness in handling, reproducibility, multi-allelic nature, codominant inheritance, relative abundance and genomic wide coverage (Powel et al., 1996). Therefore, present investigation was carried out to assess the genetic diversity using agronomical traits and molecular markers among local land races of greengram under rabi rice fallow condition.

1 Results and Discussion  The analysis of variance revealed significant differences among the 25 genotypes for all the 12 characters studied. The aggregate effect of all the 12
characters was tested by the Wilk’s criterion, indicating highly significant differences among the genotypes. Hence, the genetic divergence based on $D^2$ values was considered relevant. On the basis of $D^2$ values, the 25 genotypes were put into four clusters (Table 1). Cluster I was the largest containing 22 genotypes whereas the rest three clusters had single genotype each. The 22 genotypes of cluster I consisted of 17 land races collected from different regions of the Odisha, Kopargaon local, three commercially released varieties developed by OUAT, India and one variety developed by BARC (Bhaba Atomic Research Centre), Trombay, India. The lack of correspondence between the genetic diversity and geographical origin was observed in the present study as also reported earlier (Rahim et al., 2010; Das, 2011 and Abna et al., 2012).

![Image](https://mpb.biopublisher.ca)

Table 1 Distribution of greengram genotypes into different clusters based on $D^2$ analysis

| Cluster | No. of genotypes | Name of genotypes |
|---------|------------------|-------------------|
| I       | 22               | Tigtiria-A, Bilipara local, OUM 11-5, Keonjhar local, TARM1, Banapur local-C, Kopargaon local, OBGG 52, Nandika local, Jhain mung, Shergarh local, Khadabhanga local, Sikri local, Bijapur local, Dhauli, Bhawanipatna local-B, Kalahandi local 1-A, Kendrapara local-A, Kamakhya local-B, Sudha Sarangi local, Nayagarh local, Jharsuguda local |
| II      | 1                | Kalamuga          |
| III     | 1                | Ratila local      |
| IV      | 1                | Kalahandi local-2B |

The inter cluster distance ranged from 496.1 to 3121.6 (Table 2) indicating the clusters to be wide from each other. The maximum genetic distance was observed between cluster II and IV ($D^2$ value: 3121.6) followed by cluster III and IV ($D^2$ value: 2413.1). This indicated that Kalahandi local-2B was genetically most divergent from Kalamung followed by Ratila local and cross combination between them may yield a broad spectrum of genetic variability.

![Image](https://mpb.biopublisher.ca)

Table 2 Intra-cluster (in bold) and inter-cluster distances ($D^2$) among four clusters in greengram

| Cluster | I      | II     | III    | IV     |
|---------|--------|--------|--------|--------|
| I       | 410.5  | 496.1  | 1039.8 | 2001.9 |
| II      | 0.0    | 1095.7 | 3121.6 |
| III     | 0.0    | 2413.1 |
| IV      | 0.0    | 0.0    |

On the basis of average $D^2$ values, reaction of 10 days old plants after exposure to cold treatment contributed maximum (28.5%) towards genetic divergence followed by reaction of 40 days (28.3%) and 30 days (21.3 %) old plants on exposure to cold treatment. Clusters/ plant, pods/ plant, pod length and seeds/ pod contributed least (< 2 %) towards genetic diversity (Table 3). The moderate contributors (> 2 % but < 21.25 %) to genetic diversity were plant height, 100-seed weight, days to 50 % flowering and reaction of 20 days old plants on exposure to cold treatment. Reaction of 10, 30 and 40 days old plants on exposure to cold treatment contributed 78% of total diversity. The order of contribution of characters to $D^2$ estimates on the basis of rank average followed almost similar trend with few minor deviations. High to moderate contributions towards genetic divergence by 100-seed weight, seed yield, days to 50 % flowering and plant height have been reported earlier workers (Rahim et al., 2010 and Muhammad et al., 2007).

![Image](https://mpb.biopublisher.ca)

Table 3 Relative contribution of characters to divergence ($D^2$) in greengram

| Character                        | Average $D^2$ | Percentage of $D^2$ (%) | Rank total | Rank average (%) |
|----------------------------------|---------------|-------------------------|------------|-----------------|
| Days to 50 % flowering           | 14.864        | 2.48 (6)                | 2085       | 8.91 (6)        |
| Plant height (cm)                | 13.255        | 2.21 (8)                | 2027       | 8.66 (7)        |
| Clusters/ plant                  | 4.057         | 0.67 (11)               | 1259       | 5.38 (11)       |
| Pods/ plant                      | 6.860         | 1.14 (10)               | 1282       | 5.47 (10)       |
| Pod length (cm)                  | 8.606         | 1.43 (9)                | 2311       | 9.87 (4)        |
| Seeds/ pod                       | 2.059         | 0.34 (12)               | 1004       | 4.29 (12)       |
| 100-seed weight (g)              | 13.970        | 2.33 (7)                | 2173       | 9.28 (5)        |
| Reaction 10 days old plants to cold | 170.185     | 28.47 (1)               | 2860       | 12.22 (1)       |
| Reaction 20 days old plants to cold | 33.835      | 5.66 (5)                | 1621       | 6.92 (9)        |
| Reaction 30 days old plants to cold | 127.042     | 21.25 (3)               | 2358       | 10.07 (3)       |
| Reaction 40 days old plants to cold | 169.067     | 28.28 (2)               | 2635       | 11.26 (2)       |
| Yield/plant (g)                  | 33.859        | 5.66 (5)                | 1785       | 7.62 (8)        |
The cluster mean for the 12 characters are presented in Table 4. Cluster III had the highest yield/plant (2.7 g/plant) along with the highest cluster mean values for clusters/plant, pods/plant and seeds/plant. Cluster I had the second highest value for yield/plant (2.5 g/plant). This group had the highest mean values for 100-seed weight and moderate values for most of the traits. Cluster IV had the third highest mean value for yield/plant (1.9 g/plant) and the single genotype of this cluster had moderate value for most of the traits, except high level tolerance to cold. Cluster II had the lowest yield/plant (1.6 g/plant) but with maximum days to flowering, more plant height and moderate level of tolerance to cold.

Table 4 Character means of different clusters of greengram genotypes

| Cluster | DF    | PH   | CP    | PP    | PL    | SP    | SW    | RC10  | RC20  | RC30  | RC40  | SY    |
|---------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| I       | 40.8  | 17.7 | 3.5   | 10.5  | 8.4   | 10.1  | 2.9   | 13.8  | 33.8  | 15.3  | 15.6  | 2.5   |
| II      | 46.3  | 27.3 | 3.3   | 9.6   | 9.0   | 8.7   | 2.5   | 22.3  | 33.3  | 17.3  | 9.6   | 1.6   |
| III     | 40.0  | 16.4 | 3.7   | 12.6  | 8.5   | 10.8  | 2.4   | 20.3  | 38.0  | 46.3  | 16.0  | 2.7   |
| IV      | 42.3  | 22.6 | 4.3   | 9.7   | 8.0   | 8.7   | 2.5   | 4.0   | 38.7  | 19.3  | 44.0  | 1.9   |

Note: DF: Days to 5% flowering; PH: Plant height; CP: Clusters/plant; PP: Pods/plant; PL: Pod length; SP: Seeds/pod; SW: 100-seed weight; RC10: Reaction 10 days old plants to cold; RC20: Reaction 20 days old plants to cold; RC30: Reaction 30 days old plants to cold; RC40: Reaction 40 days old plants to cold; SY: Yield/plant

For deriving high yielding segregants, the parental genotypes should have moderately high inter cluster distance, moderate to high mean values for yield and component traits. On the basis of this rational, cross between genotypes belonging to cluster III (Ratila local) and cluster I (including most of the genotypes like Keonjhar Local, OBGG 52, OUM 11-5 and TARM 1) are expected to produce more transgressive segregants for yield and yield component traits in segregating generations.

1.1 Molecular analysis

Of the 44 SSR markers used to study amplification in 25 greengram genotypes, 19 markers resulted in amplification and produced 31 alleles with an average of 1.63 alleles bands per primer (Table 5). Out of 19 SSR markers showing amplification, 14 markers were taken from the related legume species azuki bean (Vigna angularis) which indicated that the sequence flanking SSR markers are conserved between related species in Vigna. The across species amplifications were also reported earlier in greengram (Kumar et al. 2002a; Kumar et al. 2002b and Souframanien and Gopalkrishna, 2009). Twelve SSR markers produced single allele, two produced two alleles and five produced three alleles which varied in size from 200 bp to 400 bp. Of the 31 number amplified bands, 29 numbers (93%) were polymorphic with an average of 1.63 polymorphic fragments per primer. Out of 19 SSR markers, 17 markers showed polymorphism across the greengram genotypes. Figure 1 shows amplification in mungbean genotypes by using the azuki bean SSR marker CEDG088 and CEDG092. The dendrogram constructed based on UPGMA analysis grouped the 25 greengram genotypes into six clusters (Figure 2) with the Jaccard’s similarity coefficient ranging from 0.42 to 0.94. Cluster I was the largest cluster comprising 16 genotypes distributed over five sub-clusters (Ia, Ib, Ic, Id and Ie). The
Table 5 List of micro satellite primer pairs which showed amplification for greengram genotypes

| Sl. No. | Primer     | Forward and reverse primer sequence | Derived from | No. of bands | No. of monomorphic allele | No. of polymorphic allele |
|--------|------------|------------------------------------|--------------|--------------|---------------------------|---------------------------|
| 1      | CFDC050    | F TCCCACCTCTCCATTACCTCCAC          | Azukibean    | 1            | -                         | 1                         |
|        |            | R GAGATATCTCTCTGGGCCAGCAAG         |              |              |                           |                           |
| 2      | CEDG008    | F AGGGCAGGTCTCCTGTTCCAAG           | Azukibean    | 1            | -                         | 1                         |
|        |            | R GCCCATATTTTTACGCCCCAC            |              |              |                           |                           |
| 3      | CEDG010    | F TGGGCTACCAACTTTTTCCTC            | Azukibean    | 1            | -                         | 1                         |
|        |            | R TGAGCGCAGATCTTCAACAG             |              |              |                           |                           |
| 4      | CEDG020    | F TATCCATACCAGCTCAAGG              | Azukibean    | 2            | 2                         | 2                         |
|        |            | R CCATACCAAGGAAGAGGG               |              |              |                           |                           |
| 5      | CEDG043    | F AGGATTGTGGTTGGTGCTAGT            | Azukibean    | 1            | -                         | 1                         |
|        |            | R ACTATTTCCAAGTGCTGGG              |              |              |                           |                           |
| 6      | CEDG086    | F GAGTTTACACAGATGGGGCTAA           | Azukibean    | 1            | -                         | 1                         |
|        |            | R AGGTCTTGGATTGACTTTGGT            |              |              |                           |                           |
| 7      | CEDG088    | F TCTTTGCTATTAGACCTTTAGCAG         | Azukibean    | 3            | -                         | 3                         |
|        |            | R TTGTGGTTTACAAAGACCCGTG           |              |              |                           |                           |
| 8      | CEDG091    | F CTGTTGGAACAAAGCAAAAGATGG         | Azukibean    | 1            | -                         | 1                         |
|        |            | R TGGGTCTTGGTGCAAAGAGAAA           |              |              |                           |                           |
| 9      | CEDG092    | F TCTTTTGGTTGTAGCAAGGAAGG          | Azukibean    | 3            | -                         | 3                         |
|        |            | R TACAAGTGTATGCAACGGTGTGG          |              |              |                           |                           |
| 10     | CEDG139    | F CAAACTCCGATCGAAGGGCTTG           | Azukibean    | 3            | 1                         | 2                         |
|        |            | R GTTTCTTTCCAATCTCAGCTTGG          |              |              |                           |                           |
| 11     | CEDG154    | F GTCTTGTGTCTTCTCAATGG             | Azukibean    | 1            | -                         | 1                         |
|        |            | R CATACGCTTCCACCTCCCTGG            |              |              |                           |                           |
| 12     | CEDG228    | F GTCTTGTCCGGAAAGCTGTTC            | Azukibean    | 1            | -                         | 1                         |
|        |            | R GATCGCAAGCCTCTTTCTGC             |              |              |                           |                           |
| 13     | CEDG156    | F CGCGTATTGTGACTACTAGGTAT          | Azukibean    | 1            | -                         | 1                         |
|        |            | R CTTTATGTGTGGTGTTGGTCTTAAAGG      |              |              |                           |                           |
| 14     | CEDG248    | F CAGAAACACAAAGAGGGTCTTCG          | Azukibean    | 3            | -                         | 3                         |
|        |            | R GTGAGGTCACCTTCCTCC              |              |              |                           |                           |
| 15     | VR1        | F AGCCCTTCTGCTAGGAAAT             | Mungbean     | 1            | -                         | 1                         |
|        |            | R CTCTACCGGGTGGTGTTGG             |              |              |                           |                           |
| 16     | VR2        | F CCGCCCTCTAGTGTTGTTGG            | Mungbean     | 1            | -                         | -                         |
|        |            | R GGGGAAAGCGAAGGGTGAAGA           |              |              |                           |                           |
| 17     | VR3        | F TGTTGTTGGTGTTGTTGCTAAGA         | Mungbean     | 1            | 1                         | -                         |
|        |            | R CACGGTCTTCTGTTCTCAAA             |              |              |                           |                           |
| 18     | VR5        | F TCACAAAGGGAAGGGAAGAGA           | Mungbean     | 3            | -                         | 3                         |
|        |            | R CCCCCAGGTTGTTGTTGGA             |              |              |                           |                           |
| 19     | VR6        | F GTAGGAACCTCCTCAACAGC            | Mungbean     | 2            | -                         | 2                         |
|        |            | R GTTCCACCCTCGGGTGTTGG            |              |              |                           |                           |

Total 31 2 29
Average 1.63 - -

sub-cluster Ia consisted of maximum number of 8 genotypes followed by Ic with 3 genotypes while sub-cluster Ib and Id were with 2 genotypes each and sub-cluster Ie was with a single genotype. Cluster II consisted of 3 genotypes, clusters IV and VI having 2 genotypes each, while cluster III and V were with single genotype each.

Grouping of 25 genotypes on the basis of the SSR markers did not show any parallelism between geographical origins of the genotypes. Clustering of genotypes on the basis of the SSR markers did show consistency with cluster analysis by D² method but with certain degree of dissimilarity. The lack of similarity in grouping may be due to the fact that cluster
Figure 2 Dendrogram constructed using Jaccard’s similarity coefficient and UPGMA clustering for 25 greengram genotypes based on SSR markers

analysis by $D^2$ method are based on morphological and yield component traits of the genotype, while the polymorphic bands shown by the genotypes might be expressing some more traits in addition to the agronomical traits.

It is interesting to note that amplification products or bands of primers CEDG 043 at 300 bps, VR 5 at 400 bps, CEDG 088 at 250 bps and VR-6 at 300 bps are present in only two to three genotypes whereas, CEDG 091, CEDG 228 and CEDG 248 primers at 250 bps having no bands in only two-three genotypes. Such presence/absence of an allele in any genotypes would help in DNA finger printing and molecular characterization of genotypes or varieties. Similar unique presence or absence of amplification products in varieties have been reported earlier in blackgram (Souframanien and Gopalkrishna, 2009) and in greengram (Reddy et al., 2008).

The results of this study indicate that both agronomical data and SSR markers were highly effective in estimating the genetic diversity. The SSR markers derived from other related Vigna species like azuki bean could be effectively used for molecular studies in greengram for which available SSRs are fewer. The results of this study also showed that sufficient variability exists in the local land races of greengram which can be used as parents in a breeding programme for rabi rice fallow conditions.

2 Materials and Methods

2.1 Plant material

In the present study 21 local land races along with four standard varieties of greengram, (Table 1) were evaluated in a three-row plot of 2.8m length in Randomized Block Design (RBD) with three replications at a row-to-row and plant-to-plant spacing of 30 cm x 10 cm, respectively. at EB-II of Department of Plant Breeding and Genetics, OUAT, Bhubaneswar, India.

2.2 Agronomical traits

Observations were recorded on 10 randomly selected plants of each replication on 12 quantitative traits and the mean values of three replications were used for
analysis. Reaction to cold was studied on the basis of days to mortality by exposing 10, 20, 30 and 40 days old plants to 10°C temperatures in incubator. Response to cold exposer was studied under controlled condition in the S.K. Sinha Molecular Breeding Laboratory where the genotypes were exposed to 10°C temperature at 10 days, 20 days, 30 days and 40 days seedling stage. Observations were taken on number of plant wilted in each genotype every day continuously and days to survival under cold stress was recorded. The assessment of genetic divergence for all the 12 agronomical characters was done using Mahalanobis D² statistic and the genotypes were grouped in to different clusters following Tcher’s method as described by Rao (1952).

2.3 Diversity analysis using SSR markers

The plant genomic DNA was isolated from fresh 15 days old leaves of 25 greengram genotypes following CTAB method (Doyle and Doyle, 1990). For SSR analysis, 44 selected SSR primers (Chromous Biotech Pvt. Ltd.) were used for PCR amplification out of which 19 showed amplification (Table 5). SSR amplification was performed in 20 µl volume containing 50 ng/ µl genomic DNA, 0.5 Units of Taq DNA polymerase, 0.2 mM each of dNTPs, 10 pmol/ µl SSR primer in 1 x reaction buffer that contain 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5mM MgCl₂ and 0.01% gelatin. Amplification was performed in Verti 96 well Thermal Cycler of Applied Bio system. Amplification conditions were initially a denaturation at 94°C for 4 min followed by 40 cycles at 94°C for 1 min, 47°C for 1 min, 72°C for 2 min and at last 10 min at 72°C. Amplification products were mixed with 4µl of DNA loading dye and electrophoresed through an 1.2 % agarose gel using 0.5X TAE buffer (contain 100 mM Tris HCl, pH 8.0, 8.3mM glacial acetic acid, 0.5mM EDTA) at 50 volt. The gels were visualized under UV light after staining with 0.5 µg/ml ethidium bromide solution and documented using a gel documentation and image analysis system (UVI TECH, U.K.).

The SSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of the amplification intensity. Only clear and apparently unambiguous bands were scored for SSR analysis. Binary data were analyzed using the NTSYS-pc (Numerical taxonomy system, version 2.0 (Rohlf, 1990). The SIMQUAL programme was used to calculate the Jaccard’s coefficient, a common estimator of genetic identity and was calculated as follows:

\[
\text{Jaccard’s coefficient} = \frac{N_{AB}}{N_{AB} + N_A + N_B}
\]

Where, \(N_{AB}\) is the number of bands shared by samples, \(N_A\) and \(N_B\) represent amplified fragments in sample A and B, respectively. Similarity matrices generated based on Jaccard’s coefficient was utilized to construct dendograms using UPGMA (Unweighted pair group method with arithmetic average clustering method) to elucidate the diversity among the genotype studied.

Authors’ contributions

BJ and KKP carried out the overall experiment and TRD prepared the manuscript. BB supervised the experiment as Chairman of the advisory committee for the Master degree research work.

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