# Genetic Diversity Clusters in Germplasm of Cluster Bean (*Cyamopsis tetragonoloba* L., Taub), an Important Food and an Industrial Legume Crop

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## ABSTRACT

Genetic diversity in cluster bean (*Cyamopsis tetragonoloba* L.; *Fabaceae*) genotypes was studied using Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) to derive conclusions about diversity analysis in groups of accessions of a germplasm. The two methods, individually as well as cumulatively revealed the range of diversity in profiles among 104 genotypes collected from different geographical regions of India. A simulated clustering of the collected plant genotypes was divided into defined affinity groups using Structure program and the cluster analysis of molecular markers data revealed six broad sub-clusters. These results were validated with a Principal Coordinate analysis. The combined data was more informative than either of the individual method data. The diversity range was found to be wide and the presence of six broad clusters suggests the existence of many genetic lineages that can constitute useful starting points for the use of germplasm diversity in the selection and improvement of the cluster bean crop.

**Keywords:** Diversity, ISSR, NJ, PCA, RAPD.

## INTRODUCTION

Cluster bean or guar is a drought-tolerant, annual, self pollinated, diploid (2x= 14) legume crop (Ayyangar and Krishnaswami, 1933). It has been cultivated in India and Pakistan for many centuries. Guar has many facets used for: (i) Within a crop rotation cycle; (ii) Human and animal nutrition, and (iii) As a source of galactomannan, an edible gum and categorized as an industrial crop. In fact, much more than its use as a food crop, it is best valued for the gelling agent galactomannan (guar gum) most significantly used in several different industries. Over 75% of world production of cluster bean is from India (Punia *et al.*, 2009a). However, due to the increasing demand for the plant, it is now also introduced into newer areas for cultivation. In India, though the plant is cultivated in several states, Rajasthan, Haryana, Punjab and Gujarat however, account for over 80% of the total production of cluster bean in the country. Elsewhere in the world, cluster bean is cultivated in Pakistan, Sudan, and USA. In its native range in the country, the plant does not seemingly grow in the wild states. Under cultivation, the cluster bean crop season is ~16 weeks of basically a warm weather plus moderate rainfall duration. The cluster bean however, is also well adapted to cultivation in semi-arid to arid climate zones in India (Dwivedi *et al.*, 1999; Henry and Mathur 2005; Pathak *et al.*, 2010). Basic information of genetic and phenotypic variability, heritability, genetic

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advance, correlation and direct path effects of yield related traits (Patil, 2014b), genetic components of variance (genotypic, phenotypic and error variance) and genotypes×sowing dates interaction (Patil, 2014a) were established which could facilitate the improvement of varieties and help to select a suitable breeding procedure for semi arid tract of Deccan plateau region belonging to Western Ghats of Maharashtra, India. Despite the economic importance of cluster bean, studies on genetic diversity analysis have been limited. Fifteen genotypes of cluster bean were examined for genetic diversity using three RAPD primers (Ajit *et al*., 2013) while Sharma *et al*. (2014) analyzed 35 genotypes with 20 RAPD and 10 ISSR primers that revealed ~90 and 97% polymorphic bands respectively. Prior to these, Punia *et al*. (2009a) and Pathak *et al*. (2010) analyzed 34 and 32 genotypes with 10 and 5 RAPD primers respectively. Since these earlier studies on cluster bean have been carried out with limited genotypes, they will not reflect a true extent of genetic diversity. In the present study, a wide diversity was reported by analysis of 104 genotypes of cluster beans using ISSR and RAPD profiles. Furthermore, in order to estimate the number of sub-populations among the collected *C. tetragonoloba* germplasm, population structure was simulated using model-based clustering method implemented in the program Structure and six distinct lineages were determined supporting the assessment of a wide range of genetic diversity among these genotypes.

## MATERIALS AND METHODS

### Plant Material

A total of 104 genotypes of cluster beans were collected from different geographical regions of India for the assessment of molecular genetic diversity which have been listed in Table 1.

### Isolation and Purification of Genomic DNA

Total genomic DNA from the young leaves was isolated following the Thompson and Murray (1980) method using Cetyl Trimethyl Ammonium Bromide (CTAB).

### Quantification of DNA

The quality and quantity of isolated DNA samples were checked by measuring absorbance at 260 and 280 nm using a nano-drop spectrophotometer (ND 1000). Additionally DNA samples were electrophoresed on 0.8% (w/v) agarose gel at 75V for 3-5 hours in TBE (0.5X) buffer pH 8.3 to check for the size of the isolated DNA.

### ISSR Primers and PCR Conditions

A number of ISSR primers were available. Therefore, pilot experiments were carried out with small sets of cluster bean DNAs and as many as 50 ISSR primers were screened for consistent, well banded, reproducible profiles in triplicate experiments. Additionally, in these pilot reactions, the annealing temperatures for the ISSR primers were also optimized and the final reactions were always carried out at these optimized temperatures. Only those primers resulted in discrete profiles of separated bands, which were selected for analysis of full set of cluster bean genotypes (Table 2). Typical ISSR reaction consisted of 50 ng DNA, 10 pmoles primers, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 µM each dNTP and 0.75 U of *Taq* DNA Polymerase (Bangalore Genei, Bangalore, India) in a final volume of 25 µl; while the PCR parameters included pre-denaturation at

**Table 1**. The cluster bean genotypes collected from different locations in the country. All samples were collected or procured as seed packets. The seeds were planted in a well spaced out of the field plot in the botanical garden of the Institute and fresh healthy leaf tissue were harvested for the DNA isolation.

| Sample no. | Genotype | Collected from | Sample no. | Genotype | Collected from |
|---|---|---|---|---|---|
| Ct1 | 325806 | CAZRI, Jodhpur | Ct53 | 311442 | CAZRI, Jodhpur |
| Ct2 | 329036 | CAZRI, Jodhpur | Ct54 | 415162 | CAZRI, Jodhpur |
| Ct3 | 329030 | CAZRI, Jodhpur | Ct55 | 323992 | CAZRI, Jodhpur |
| Ct4 | 370478 | CAZRI, Jodhpur | Ct56 | 373438 | CAZRI, Jodhpur |
| Ct5 | 373557 | CAZRI, Jodhpur | Ct57 | 311393 | CAZRI, Jodhpur |
| Ct6 | 415342 | CAZRI, Jodhpur | Ct58 | 373467 | CAZRI, Jodhpur |
| Ct7 | 402298 | CAZRI, Jodhpur | Ct59 | 415145 | CAZRI, Jodhpur |
| Ct8 | 421810 | CAZRI, Jodhpur | Ct60 | 329062 | CAZRI, Jodhpur |
| Ct9 | 258101 | CAZRI, Jodhpur | Ct61 | 311405 | CAZRI, Jodhpur |
| Ct10 | 329030 LT | CAZRI, Jodhpur | Ct62 | 311449 | CAZRI, Jodhpur |
| Ct11 | 329030 SPS Trial | CAZRI, Jodhpur | Ct63 | 311444 | CAZRI, Jodhpur |
| Ct12 | 311406 | CAZRI, Jodhpur | Ct64 | 325743 | CAZRI, Jodhpur |
| Ct13 | 370509 | CAZRI, Jodhpur | Ct65 | 415157 | CAZRI, Jodhpur |
| Ct14 | 369789 | CAZRI, Jodhpur | Ct66 | 311414 | CAZRI, Jodhpur |
| Ct15 | 415166 | CAZRI, Jodhpur | Ct67 | 370502 | CAZRI, Jodhpur |
| Ct16 | 311433 LT | CAZRI, Jodhpur | Ct68 | 369868 | CAZRI, Jodhpur |
| Ct17 | Selection-1 | Lucknow | Ct69 | 415153 | CAZRI, Jodhpur |
| Ct18 | RGC-1033 | CAZRI, Jodhpur | Ct70 | 311392 | CAZRI, Jodhpur |
| Ct19 | HG-563 | Lucknow | Ct71 | 370468 | CAZRI, Jodhpur |
| Ct20 | RGC-197 | CAZRI, Jodhpur | Ct72 | 373480 | CAZRI, Jodhpur |
| Ct21 | RGC-1055 | CAZRI, Jodhpur | Ct73 | 324023 | CAZRI, Jodhpur |
| Ct22 | Pusa Navbahar | Lucknow | Ct74 | RGC-1002 PAS | CAZRI, Jodhpur |
| Ct23 | RGC-471 | CAZRI, Jodhpur | Ct75 | RGC-1066 | RARI, Jaipur |
| Ct24 | RGC-986 | RARI, Jaipur | Ct76 | Sharda | Lucknow |
| Ct25 | RGC-1038 | CAZRI, Jodhpur | Ct77 | Raipur hybrid | Lucknow |
| Ct26 | RGC-1003 | RARI, Jaipur | Ct78 | RGC-1038 | RARI, Jaipur |
| Ct27 | Jaisalmer local | SKRAU, Bikaner | Ct79 | RGC-936 | CAZRI, Jodhpur |
| Ct28 | RGC-1066 | CAZRI, Jodhpur | Ct80 | Gst 13-1 | SV University, Tirupati |
| Ct29 | RGC-1017 | RARI, Jaipur | Ct81 | Gst 13-19 PAS | SV University, Tirupati |
| Ct30 | RGC-1002 | CAZRI, Jodhpur | Ct82 | Gst 13-13 | SV University, Tirupati |
| Ct31 | Gst 13-11 | SV University, Tirupati | Ct83 | Gst 13-18 | SV University, Tirupati |
| Ct32 | Gst 13-10 | SV University, Tirupati | Ct84 | Gst 13-17 | SV University, Tirupati |
| Ct33 | Gst 13-9 | SV University, Tirupati | Ct85 | Gst 13-16 | SV University, Tirupati |
| Ct34 | Gst 13-8 | SV University, Tirupati | Ct86 | Gst 13-15 | SV University, Tirupati |
| Ct35 | Gst 13-7 | SV University, Tirupati | Ct87 | Gst 13-14 | SV University, Tirupati |
| Ct36 | Gst 13-6 | SV University, Tirupati | Ct88 | Gst 13-12 | SV University, Tirupati |
| Ct37 | Gst 13-5 | SV University, Tirupati | Ct89 | 324008 | CAZRI, Jodhpur |
| Ct38 | Gst 13-4 | SV University, Tirupati | Ct90 | 370490 | CAZRI, Jodhpur |
| Ct39 | Gst 13-3 | SV University, Tirupati | Ct91 | 420332 | CAZRI, Jodhpur |
| Ct40 | Gst 13-2 | SV University, Tirupati | Ct92 | 415112 | CAZRI, Jodhpur |
| Ct41 | Lucknow local | Lucknow | Ct93 | 311422 | CAZRI, Jodhpur |
| Ct42 | Gst 13-19 | SV University, Tirupati | Ct94 | 311407 | CAZRI, Jodhpur |
| Ct43 | Desi Anupama | Lucknow | Ct95 | 402294 | CAZRI, Jodhpur |
| Ct44 | Bhusawal local | Lucknow | Ct96 | 311438 | CAZRI, Jodhpur |
| Ct45 | RGC-936 | RARI, Jaipur | Ct97 | 373427 | CAZRI, Jodhpur |
| Ct46 | RGC-1031 | RARI, Jaipur | Ct98 | 415148 | CAZRI, Jodhpur |
| Ct47 | Swati-55 | Lucknow | Ct99 | 415131 | CAZRI, Jodhpur |
| Ct48 | Raipur local | Lucknow | Ct100 | 311428 | CAZRI, Jodhpur |
| Ct49 | Kanpur local | Lucknow | Ct101 | 415111 | CAZRI, Jodhpur |
| Ct50 | 311401 | CAZRI, Jodhpur | Ct102 | 415102 | CAZRI, Jodhpur |
| Ct51 | 415160 | CAZRI, Jodhpur | Ct103 | Surat local | Surat, Gujarat |
| Ct52 | 369861 | CAZRI, Jodhpur | Ct104 | Omaxe parth hybrid | Jabalpur, Madhya Pradesh |
| | | | OG-1 | Outgroup (*Clitorea ternatea*) | Lucknow |

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**Table 2.** The different primers, their sequences and annealing temperatures used for ISSR and RAPD methods are listed. The primers were selected for amplifying all the *C. tetragonoloba* genotype DNAs after screening in pilot reactions as described.

| Primer | Sequence (5' – 3') | Annealing Temperature (°C) [c] | Numbers of bands (T= Total; P= Polymorphic, M= Monomorphic) [c] | | | Proportion of P bands (%) |
|---|---|---|---|---|---|---|
| | | | T | P | M | |
| 808 [a] | AGAGAGAGAGAGAGAGC | 51 [e] | 9 | 9 | 0 | 100 |
| 809 [a] | AGAGAGAGAGAGAGAGG | 46 [e] | 8 | 8 | 0 | 100 |
| 823 [a] | TCTCTCTCTCTCTCC | 52 [e] | 12 | 11 | 1 | 92 |
| 824 [a] | TCTCTCTCTCTCTCG | 51 [e] | 5 | 5 | 0 | 100 |
| 825 [a] | ACACACACACACACACT | 42 [e] | 9 | 9 | 0 | 100 |
| 855 [a] | ACACACACACACACACYT | 54 [e] | 15 | 15 | 0 | 100 |
| 866 [a] | CTCCTCCTCCTCCTCCTC | 51 [e] | 11 | 10 | 1 | 91 |
| 868 [a] | GAAGAAGAAGAAGAAGAA | 46 [e] | 9 | 9 | 0 | 100 |
| 880 [a] | GGAGAGGAGAGGAGA | 42 [e] | 14 | 14 | 0 | 100 |
| 888 [a] | (B)(D)(B)CACACACACACA | 51 [e] | 10 | 9 | 1 | 90 |
| | | | **102** | **99** | **3** | **97.3** |
| OP-F02 [b] | GAGGATCCCT | 35 | 16 | 16 | 0 | 100 |
| OP-F04 [b] | GGTGATCAGG | 35 | 16 | 16 | 0 | 100 |
| OP-F05 [b] | CCGAATTCCC | 35 | 14 | 11 | 3 | 79 |
| OP-F09 [b] | CCAAGCTTCC | 35 | 11 | 6 | 5 | 55 |
| OP-F18 [b] | TTCCCGGGTT | 35 | 17 | 17 | 0 | 100 |
| OP-G04 [b] | AGCGTGTCTG | 35 | 14 | 13 | 1 | 93 |
| OP-G13 [b] | CTCTCCGCCA | 35 | 14 | 10 | 4 | 71 |
| OP-W04 [b] | CAGAAGCGGA | 35 | 12 | 12 | 0 | 100 |
| OP-W10 [b] | TCGCATCCCT | 35 | 11 | 9 | 2 | 82 |
| OP-W15 [b] | ACACCGGAAC | 35 | 14 | 13 | 1 | 93 |
| | | | **139** | **123** | **16** | **87.3** |

[a] ISSR Primers; [b] RAPD primers, [c] Annealing temperatures for ISSR primers were optimized separately in pilot experiments while those for the RAPD primers were always set at 35°C. The total numbers of bands and proportion of polymorphic bands for ISSR and RAPD primers respectively are indicated in bold fonts.

$94^{\circ}$C for 5 minutes, followed by 45 cycles of denaturation at $94^{\circ}$C for 45 seconds, annealing at optimized temperature for 45 seconds and extension at $72^{\circ}$C for 2 minutes. The final cycle allowed an additional extension at $72^{\circ}$C for 5 minutes.

## RAPD Primers and PCR Conditions

Pilot experiments were carried out with small sets of cluster bean DNAs and as many as 60 RAPD primers were screened (20 primers from each of the three kits, F, G and W; QIAGEN OPERON primers) for consistent, well banded, reproducible profile in triplicate experiments. The typical RAPD reaction consisted of 50 ng DNA, 10pmoles primers, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 µM each dNTP and 0.75 U of *Taq* DNA Polymerase (Bangalore Genei, Bangalore, India) in a final volume of 25 µl; while the PCR parameters included pre-denaturation at $94^{\circ}$C for 3 minutes, followed by 45 cycles of denaturation at $94^{\circ}$C for 1 minute, annealing at $35^{\circ}$C for 1 minute and extension at $72^{\circ}$C for 1.5 minutes. The final cycle allowed an additional extension at $72^{\circ}$C for 5 minutes.

## Agarose Gel Electrophoresis

The PCR products were electrophoresed on agarose gels 1.2% (w/v) in 0.5X TBE buffer, pH 8.3 and containing ethidium bromide (0.5 µg $ml^{-1}$) at a constant 75 volt. After electrophoresis, the gels were viewed on UV transilluminator and images were saved using the Alpha Imager gel documentation system. The low range DNA ruler was used as the DNA size marker for comparing the profiles among the different genotypes of cluster bean.

## Band Scoring and Data Analysis

Clear and well separated bands were coded in a binary form by denoting '0' and '1' for absence and presence of bands, respectively in each genotype and for each primer. For a given method bands from all primers were scored cumulatively. Additionally a cumulative data matrix was generated by combining RAPD and ISSR data. The band data were analyzed for pair wise distance using the Jaccard coefficient in Free Tree Package (Pavlicek *et al.*, 1999). Likewise, the 1,000 replicate bootstrap tree was also generated using the same package by the NJ method. The tree files were viewed, annotated and printed using the Tree View program (Page, 2001). For an estimation of the number of sub-populations among the collected *C. tetragonoloba* germplasm, population structure simulation analysis was carried out using model-based clustering method implemented in the Program Structure, version 2.3.4 (Pritchard *et al.*, 2000). The membership of each genotype was tested for the range of genetic clusters from $K= 2$ to 10 with admixture model and without prior information on their origin. Three independent runs were assessed for each labeled $K$ and each run consisted of 30,000 burn-in period and 100,000 iterations. The optimal value of $K$ was determined by examination of the $\Delta K$ statistic and $L$ (K) according to Evanno *et al.* (2005) using the program Structure Harvester (Earl and von Holdt, 2012). The Principle Coordinate plot was generated using the program GenAlEx, ver. 6.5 (Peakall and Smouse, 2012).

## RESULTS

The total genomic DNAs were prepared from leaf tissues as described. The DNA was found to be high molecular weight (as determined by agarose gel electrophoresis, data not shown), and had spectrophotometer ratios A260/A280 in the range of 1.82-2.17 determined by Nano-Drop 1000 spectrophotometer. Between three and five DNAs were randomly selected to set up pilot reaction for screening ISSR and RAPD

primers as described. On the basis of the pilot experiments, primers that resulted in distinct well separated bands in case of all the genotype clustered were selected for further analysis and these primers have been listed in Table 2. In the above pilot experiment DNA from out-group taxon (Table 1) was also processed under similar conditions to that of cluster beans.

## ISSR-PCR ANALYSIS

ISSR method resolved a total of 102 bands for all the selected primers (Table 2), from which 99 bands were polymorphic (~97%). The 10 primers individually resolved between five (with primer 824) and 15 (with primer 855) bands across all the genotypes. Seven of the ten primers revealed 100% polymorphic bands and only the primers 823, 866 and 888 revealed mono-morphic bands as well (Table 2). Band data obtained in case of all the ISSR primers for the cluster bean genotypes were input as the binary matrix of "1" and "0" as described. From this binary matrix pair wise, Jaccard distances were computed. The range of distances between pairs of cluster bean genotypes were found to be 0 (Ct67 and Ct69) to 0.90 (Ct64 and Ct104) with an average distance across all the pairs of 0.55 (Table 3). The NJ dendrogram resolved all the cluster bean genotypes into broad sub-clusters with the out-group taxa clearly separated from the cluster bean genotypes (Figure 1-a).

## RAPD-PCR Analysis

RAPD method resolved a total of 139 bands for all the selected primers (Table 2), from which 123 bands were polymorphic (~87%). The 10 primers individually resolved between 11 (with primer OP-F09 and OP-W10) and 17 (with primer OP-F18) bands across all the genotypes. Unlike ISSR primers, only four (OP-F02, OP-F04, OP-F18 and OP-W04) of the ten primers revealed 100% polymorphic bands while the remaining primers revealed monomorphic bands as well (Table 2). Band data obtained in case of all the ISSR primers for the cluster bean genotypes were input as the binary matrix of "1" and "0" as described. From this binary matrix pair wise, Jaccard distances were computed. The distances between pairs of cluster bean genotypes were found to be in the range 0.024 (Ct87 and Ct91) to 0.68 (Ct57 and Ct104) with an average distance across all the pairs of 0.41 (Table 3). Similarly in this case, the NJ dendrogram resolved all the cluster bean genotypes into broad sub-clusters with the out-group taxa clearly separated from the cluster bean genotypes (Figure 1-b).

## Cumulative Band Data

In order to better resolve the affinities of genotypes to each other the ISSR and RAPD data were considered cumulatively. Here the least (0.023) and highest (0.75) pair wise distances were found to be between genotypes Ct67, Ct69, Ct64 and Ct104

**Table 3.** Pairwise Jaccard distances were computed as described for the band data of each method separately as well as for the combined data of the two methods. The ISSR method revealed a wider range of distances while the RAPD method revealed the smallest range.

| Method | Jaccard Distances | | | Δ Range of distances |
|---|---|---|---|---|
| | Minimum | Maximum | Average | |
| ISSR | 0.00 | 0.90 | 0.55 | 0.90 |
| RAPD | 0.024 | 0.68 | 0.41 | 0.56 |
| ISSR + RAPD | 0.023 | 0.75 | 0.47 | 0.63 |
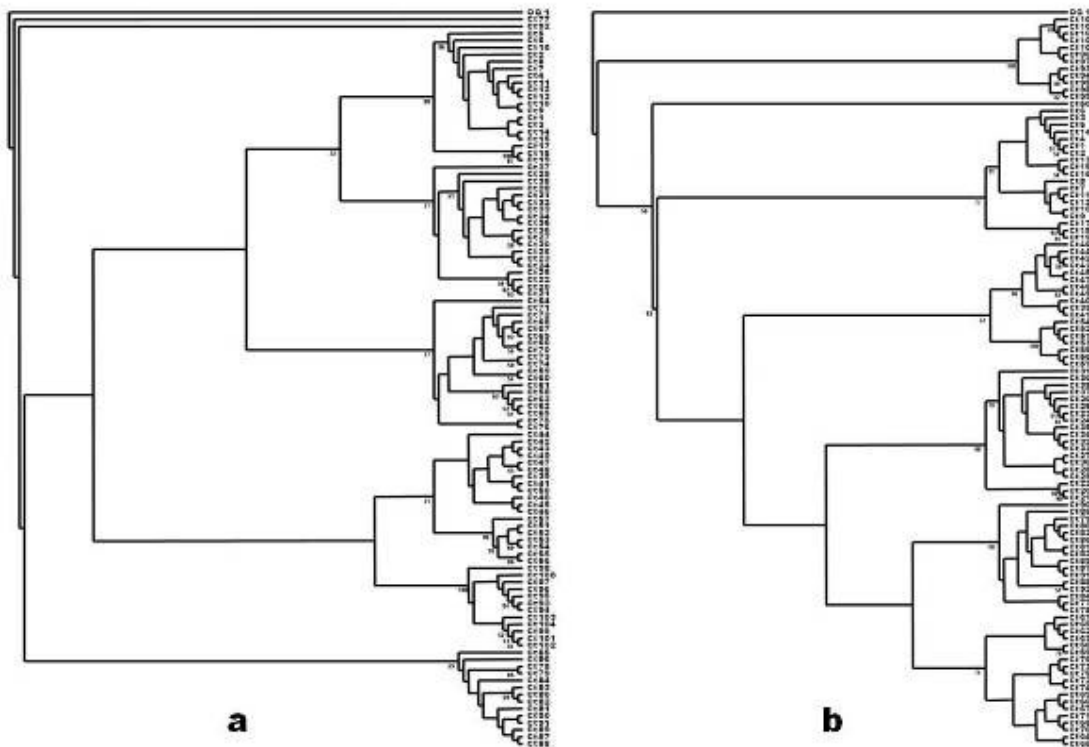
**Figure 1.** NJ trees for ISSR **(a)** and RAPD **(b)** primer data. The band data for the two methods were analyzed by FREETREE and NJ trees were computed on the basis of pair wise Jaccard distances. The generated tree files were viewed and annotated by TREE View program. The OTU names in the trees are the *Cyamopsis tetragonoloba* genotypes and out-group as in Table 1. The lengths of the branches in each tree are scaled according to the Jaccard distances.

respectively, while the average distance across all the pairs was 0.47 (Table 3). The NJ dendrogram, after a 1,000 replicate bootstrap is shown in Figure 3-a. The out-group taxa were well separated from the rest of the cluster bean genotypes which were in turn grouped into six clusters marked with colored bars.

## Model-based Structure Simulation Analysis

The observation that the combined ISSR and RAPD data resolved an NJ tree with all genotypes divided in six broad clusters suggests that these clusters represent many genetic lineages. The NJ tree however, does not reveal genetic admixtures, if any, among the genotypes and in order to assess this, a

simulation was carried out using model-based clustering in the Structure program where data were analyzed for membership of each genotype in two to ten possible clusters allowing admixtures. This analysis of simulation with each of the ten values of $K$ revealed that the simulation of six population clusters best resolved the data in terms of least deviations in probabilities of the estimates and the highest delta $K$ values (Figures 2-a and -b). On this basis the extent of membership of each genotype in the six population clusters was plotted by assigning a different color to the population. This plot has been given in Figure 3-b along with the NJ tree (Figure 3-a) for the combined PCR profile data for comparison. Colored bars representing the total clusters in both the NJ tree as well as the structure plot are used to

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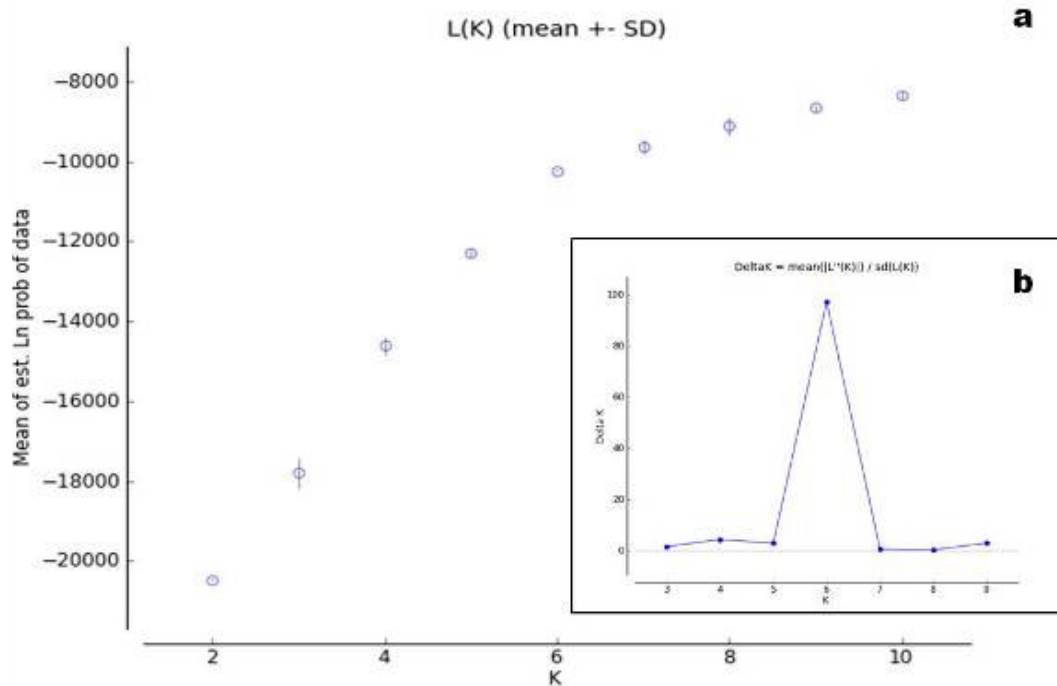
**Figure 2. (a)** Plot of *K* values with mean of estimated log probability. The small vertical bars in the plot at each K value indicate the deviation range of estimated log probability means in different replicates. **(b)** Plot of *K* values with *ΔK* generated for simulation analysis using Structure program and Structure Harvester as described. In both plots, for *K* value 6, least deviation in log probability and maximum *ΔK* indicate that this value of *K* represents the best possible numbers of clusters that the genotypes can be grouped under the conditions of the simulation carried out.

show congruence of genotypes by both methods. The six clusters identified in the present study are validated by a Principle Component Analysis where the plot of genotypes is consistent with the grouping in the Structure simulation clusters (Figure 4). The genotype groupings in the present study however, do not show any relationship to the provenance of the genotypes. Primarily the clusters data indicate that there are as many as six genetic lineages among the tested genotypes in the present study. Such a distribution of genotypes in the collection is useful to identify potentially contrasting genotypes for agro-morphological traits as well as their utilization for the subsequent cluster bean improvement program.

## DISCUSSION

The ISSR and RAPD markers, individually or together with other markers, are most widely used for identifying relationships at the cultivar and species level as well as for estimating genetic diversity in plant germplasm. These markers have been used individually as well as together and have generated many useful data about the plant genotypes studied. Though these markers were first described 20 and 24 years ago (ISSR - Zietkiewicz *et al*., 1994; RAPD - Williams *et al*., 1990; Welsh and McClelland, 1990), they are still being used with considerable success. Recent reports of the application of these markers included the

**1400**
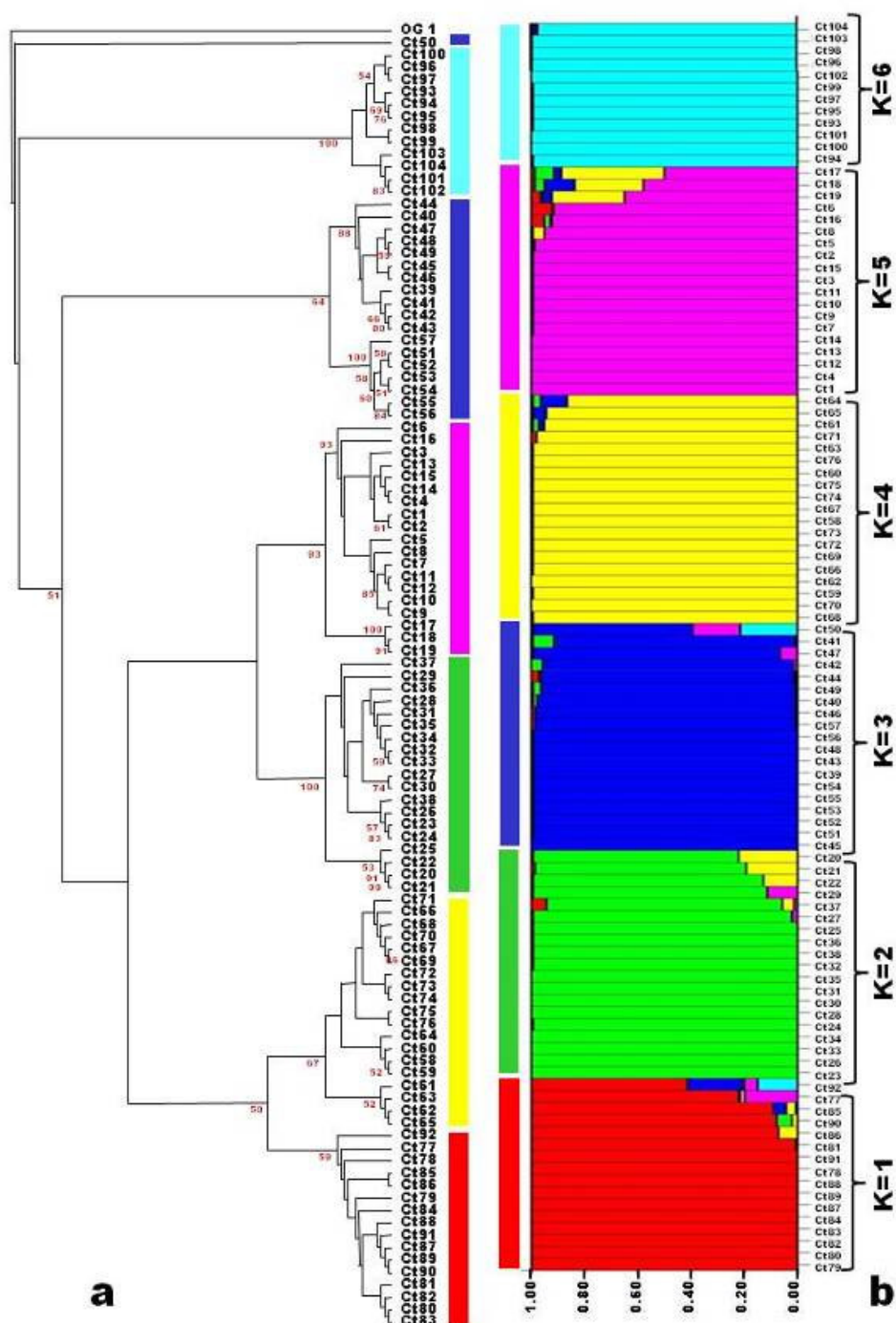
**Figure 3. (a)** The NJ tree from the combined band data of both ISSR and RAPD primers. The tree was generated after a 1000 replicate bootstrap and only 50% or greater bootstrap supports are indicated for clarity (small numbers before the nodes). The tree indicates resolution of all the *Cyamopsis tetragonoloba* genotypes into six broad clusters. The genotypes are identified by numbers as in Table 1. **(b)** The *K* plot of all genotypes in a model of six clusters (designated as K= 1 to K =6) is depicted in this figure. The genotypes are identified by numbers as in Table 1. The cluster distribution of the genotypes is color coded with six colors to further depict admixtures, if any. Similar color bars corresponding to the total clusters in both NJ tree as well as the *K* plot have been provided such that the color coding enables easy identification of the clusters congruent in both plots.

**1401**
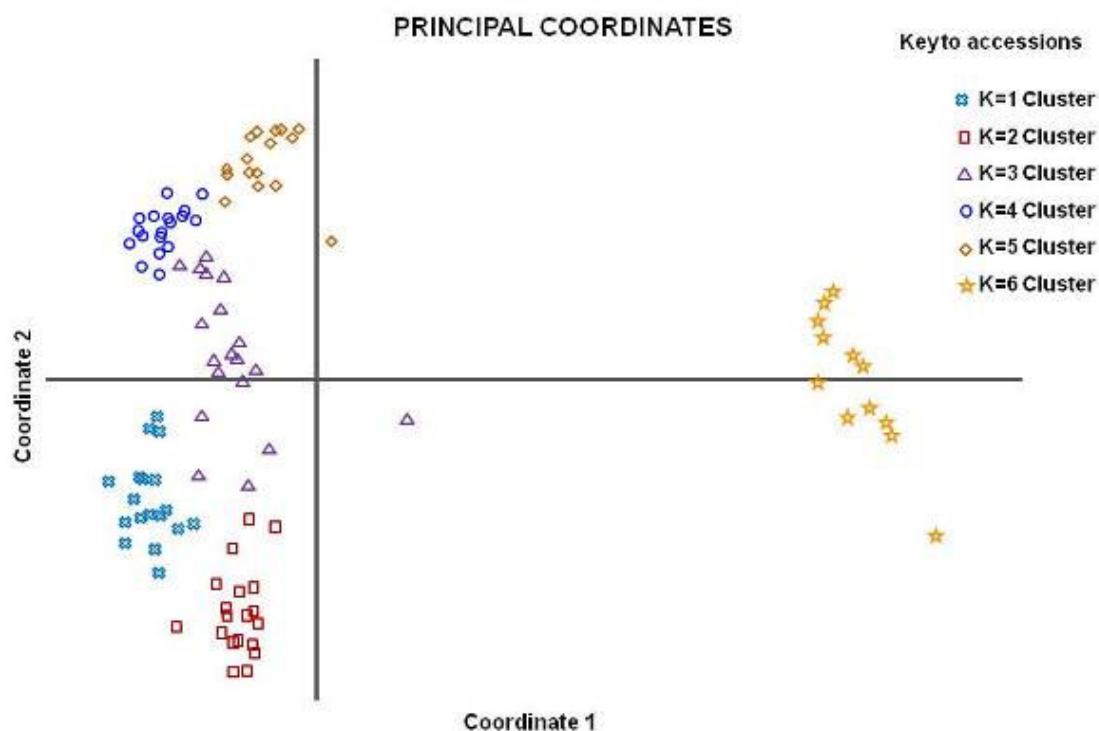
**Figure 4.** Principle Coordinate Analysis plot for 104 genotypes of cluster bean considered as belonging to the six clusters (K= 1 to K= 6) according to the Structure simulation. Consistent with the simulation, very little admixture is found among the genotypes. The genotypes in a given cluster share the same symbol as shown in the figure key.

assessment of genetic diversity in plants like sugarcane (Khaled *et al*., 2015); castor (Kallamadi *et al*., 2015); and *Aconitum* germplasm (Zhao *et al*., 2015). In a plant like *Harpagophytum*, Muzila *et al*. (2014) have used RAPD and ISSR markers to provide evidence of introgression. These two markers have also been used to assess genetic structure of olive populations (Linos *et al*., 2014) also for divergence analysis and conservation management in a medicinal plant named *Justicia adhatoda* L. (Kumar *et al*., 2014). Similarly Vaishali *et al*. (2014) have compared genetic diversity analysis of *Butea monosperma* from different agro-ecological regions of India by ISSR and RAPD marker systems. These markers have also been used in case of *Moringa oleifera* Lam. (Saini *et al*., 2013) cultivars from India where the authors have resolved

considerable genetic diversity among the cultivars. In another study, Jayabalan *et al.* (2013) have assessed genetic diversity in *Solanum trilobatum* L., an important medicinal plant from South India and showed that the UPGMA cluster analysis grouped all Tamil Nadu accessions in one cluster relative to accessions from other states. All of these recent reports continue to support the utility of both marker systems to resolve genetic diversity in plants. The two markers have also been employed in several legumes in the recent times for studies on diversity, population structure as well as for germplasm characterization (Cowpea – Anatala *et al*., 2014; Gajera *et al*., 2014; Khan *et al*., 2015; Pigeonpea - Yadav *et al*., 2014; Chickpea - Singh *et al*., 2014; Mung bean and other Vigna species – Das *et al*., 2014; Singh *et al*., 2014; Faba bean -

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Ammar *et al*., 2015; Cluster bean - Sharma *et al*., 2014; winged bean – Chen *et al*., 2015; horsegram – Sharma *et al*., 2015; lentils – Idrissi *et al*., 2015; tree legume – Li and Geng, 2015). The profusion of these recent studies suggests that many leguminous crops are now the subjects of investigation with molecular markers.

In case of cluster bean, the studies reported this far have not only used a limited number of genotypes but have also used only one marker type at a time (Punia *et al*., 2009b; Pathak *et al*., 2010; Ajit *et al*., 2013) and only Sharma *et al*. (2014) have used both markers. They have however, used limited genotypes and might have not represented a true extent of genetic diversity in cluster bean in the country. We have used a large number of genotypes collected from different locations and observed diversity amongst these with similarity coefficients ranging from the least 0.023 to the highest 0.75 when ISSR and RAPD data are considered together. Individually, the ISSR method revealed the most diversity. This result is in agreement with that reported earlier by Sharma *et al*. (2014) who also observed greater resolution power in the ISSR method relative to the RAPD method. However, considering that the ISSR method reveals polymorphism in the regions of genome rich in SSR while the RAPD profiles are from more dispersed regions of the genome, it is more useful to analyze the two methods in combination. In the present study a large number of genotypes were from CAZRI, Jodhpur followed by SV University, Tirupati, India (Table 1). However, the UPGMA dendrogram which revealed 5 broad clusters (Figure 1) does not show any specific grouping with reference to the provenance of the genotypes. Interestingly, both the Jodhpur and Tirupati genotypes are included among more than one cluster suggesting the genetic differences between them. A few genotypes, common to our study and those of Pathak *et al*. (2010) and Sharma *et al*. (2014), were reported as being separated broadly in two clusters (Pathak *et al*. 2010) while that of

Sharma *et al*. (2014) had shown these genotypes to be mostly in one large cluster of more than 25 genotypes and 3-4 small clusters of just 1-2 genotypes each. Interestingly our study also reveals that these genotypes are distributed separately in the different sub-clusters. Thus the use of the two primers in our study has enabled a better distinction among these genotypes than those resolved by Pathak *et al*. (2010) and Sharma *et al*. (2014). Furthermore, even though a set of genotypes has been obtained from a single source collection, there is sufficient diversity among the genotypes which are grouped separately from each other. The geographical provenances (source collections) do not always correlate with genetic diversity. Earlier this was shown in case of Mung bean (Lavanya *et al*., 2008; Lavanya and Ranade, 2013) while the converse has been shown by Jayabalan *et al*. (2013) in case of a medicinal plant named *Solanum trilobatum*. In general, it can be stated that a majority of the studies using ISSR and RAPD markers have resolved the genotype collections tested in two or more discrete sub-clusters that may or may not reflect geographical provenance, but they are more due to the many genetic lineages among the collections.

The results of this study with a large number of cluster bean genotypes and using two methods suggests that: (i) The individual methods are useful for diversity analysis among the genotypes; (ii) When data from both methods are considered together it is more informative; (iii) The distribution of genotypes in clusters is not correlated to their provenance, and (iv) The broad clusters of genotypes in previous studies were better separated in the present study. Thus these results clearly suggest that the methods are not only useful in revealing diversity among the genotypes, but also suggest the existence of sufficient diversity among them to identify promising genotypes as an invaluable genetic resource for the improvement of cluster bean genotypes. Consistent with the increasing application of molecular markers, we find that the use of

two markers together is more useful for the assessment of diversity in cluster bean as well, as was observed in several legumes stated above. The observed diversity is a useful range to select specific genotypes for agro-physiological traits such as drought resistance as well as for economic traits such as yields and gum quality and quantities amongst these genotypes for a sustained selection and breeding program in cluster bean.

## Abbreviations

ISSR: Inter-Simple Sequence Repeat, NJ: Neighbor Joining, RAPD: Random Amplified Polymorphic DNA.

## ACKNOWLEDGEMENTS

## REFERENCES

1. Ajit, P., Yengkokpam, P., More, V., Ghorpade, B. and Swamy, M. K. 2013. Molecular Characterization of Cluster Bean (*Cyamopsis tetragonoloba*) Cultivars Using PCR-based molecular markers. *Int. J. Adv. Biotechnol. Res.,* **4**: 158-166.
2. Ammar, M. H., Alghamdi, S. S., Migdadi, H. M., Khan, M. A., El-Harty, E. H. and Al-Faifi, S. A. 2015. Assessment of Genetic Diversity among Faba Bean Genotypes Using Agro-morphological and Molecular Markers. *Saudi J. Biol. Sci.,* **22**: 340–350.
3. Anatala, T. J., Gajera, H. P., Savaliya, D. D., Domadiya, R. K., Patel, S. V. and Golakiya, B. A. 2014. Molecular Diversity Analysis of Cowpea (*Vigna unguiculata* L.) Genotypes Determined by ISSR and RAPD Markers. *IJAEB,* **7**: 269 - 276.
4. Ayyangar, G. N. P. and Krishnaswami, N. 1933. A note on the Chromosome Number in Cluster Bean (*Cyamopsis psoralioides* DC). *Indian J. Agr. Sci.,* **3**: 934 - 935.
5. Chen, D., Yi, X., Yang, H., Zhou, H., Yu, Y., Tian, Y. and Lu, X. 2015. Genetic Diversity Evaluation of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) Using Inter-Simple Sequence Repeat (ISSR). *Genet. Resour. Crop Ev.*, DOI: 10.1007/s10722-015-0261-3
6. Das, S., Das, S. S. and Ghosh, P. 2014. A Comparative Analysis of Genetic Diversity across Certain Mung Bean and Urd Bean Cultivars of West Bengal, Using ISSR Markers. *Asian J. Plant Sci. Res.,* **4**: 56 - 61.
7. Dwivedi, N. K., Bhandari, D. C., Bhatnagar, N. and Dabas, B. S. 1999. Characterization of Cluster Bean [*Cyamopsis tetragonoloba* (L) Taub.] Germplasm for Yield and Quality Traits. *Ann. Arid Zone,* **38**: 151-156.
8. Earl, D. A. and von Holdt, B. M. 2012. Structure Harvester: A Website and Program for Visualizing Structure Output and Implementing the Evanno Method. *Conserv. Genet. Resour.,* **4**: 359–361.
9. Evanno, G., Regnaut, S. and Goudet, J. 2005. Detecting the Number of Clusters of Individuals Using the Software Structure: A Simulation Study. *Mol. Ecol.*, **14**: 2611–2620.
10. Gajera, H. P., Domadiya, R. K., Patel, S. V. and Golakiya, B. A. 2014. Appraisal of RAPD and ISSR Markers for Genetic Diversity Analysis among Cowpea (*Vigna unguiculata* L.) Genotypes. *J. Crop Sci. Biotechnol.,* **17**: 79 - 88.
11. Henry, A. and Mathur, B. K. 2005. Genetic Diversity and Performance of Cluster Bean Varieties for Stability and Quantitative Characters in Arid Region. *J. Arid Legum.,* **2**: 145 - 148.
12. Idrissi, O., Udupa, S. M., Houasli, C., De Keyser E., Van Damme, P. and De Riek, J. 2015. Genetic Diversity Analysis of Moroccan Lentil (*Lens culinaris* Medik.) Landraces Using Simple Sequence Repeat and Amplified Fragment Length Polymorphisms Reveals Functional Adaptation towards Agro-environmental Origins. *Plant Breed.,* **134**: 322 – 332.
13. Jayabalan, S., Tamilarasan, S., Shunmugiah, K. P. and Manikandan, R. 2013. Assessment of Genetic Diversity in *Solanum trilobatum* L., an Important Medicinal Plant from South India Using RAPD and ISSR Markers. *Genet. Resour. Crop Ev.*, **60**: 807 - 818.
14. Kallamadi, P. R., Nadigatla, V. G. R. and Mulpuri, S. 2015. Molecular Diversity in Castor (*Ricinus communis* L.). *Ind. Crop. Prod.,* **66**: 271 - 281.
15. Khaled, K. A., El-Demardash, I. S. and Amer, E. A. M. 2015. Genetic Polymorphism among Some

Sugarcane Germplasm Collections as Revealed by RAPD and ISSR Analysis *Life Sci. J.,* **12**: 159 -167.

16. Khan, Md. T., Reza, Md. O. H., Khan, Md. A., Haque, Md. S., Islam, Md. S. and Khan, Md. B. 2015. Genetic Diversity Analysis of Cowpea by RAPD Markers. *IJIAS,* **10**: 459 - 465.

17. Kumar, A., Mishra, P., Singh, S. C. and Sundaresan, V. 2014. Efficiency of ISSR and RAPD Markers in Genetic Divergence Analysis and Conservation Management of *Justicia adhatoda* L., a Medicinal Plant. *Plant Syst. Evol.*, **300**: 1409 -1420.

18. Lavanya, G. R. and Ranade, S. A. 2013. Comparative Analysis of Morphological and Molecular Diversity in Mung bean (*Vigna radiata* L. Wilczek). *Trend. Biosci,.* **6**: 146 - 151.

19. Lavanya, G. R., Srivastava, J. and Ranade, S. A. 2008. Molecular Assessment of Genetic Diversity in Mung Bean Germplasm. *J. Genet.*, **87**: 65 - 74.

20. Li, H. and Geng, S. 2015. Assessment of Population Genetic Diversity of *Derris elliptica* (Fabaceae) in China Using Micro Satellite Markers. *Ind. Crop. Prod.*, **73**: 9 - 15.

21. Linos, A., Nikoloudakis, N., Katsiotis, A. and Hagidimitriou, M. 2014. Genetic structure of the Greek Olive Germplasm Revealed by RAPD, ISSR and SSR Markers. *Sci. Hortic. (Amsterdam),* **175**: 33 - 43.

22. Muzila, M. M., Werlemark, G., Ortiz, R., Sehic, J., Fatih, M., Setshogo, M., Mpoloka, W. and Nybom, H. 2014. Assessment of Diversity in *Harpagophytum* with RAPD and ISSR Markers Provides Evidence of Introgression. *Hereditas,* **151**: 91 - 101.

23. Page, R. D. M. 2001. *Tree View (Win 32) Ver. 1.6.5.* Distributed by Author, http://taxonomy.zoology.gla.ac.uk/rod/rod.html

24. Pathak, R., Singh, S. K., Manjit, S. and Henry, A. 2010. Genetic Divergence in Cluster Bean (*Cyamopsis tetragonoloba*) for Seed Yield and Gum Content under Rain-fed Conditions. *Indian J. Agr. Sci.* **79**, 559 - 561.

25. Patil, D.V. 2014a. Genetic Components of Variance of Guar and Its Response to Sowing Dates in Semi Arid Region of Maharashtra, India. *Plant Archiv.,* **14(1):** 125 – 131.

26. Patil, D. V. 2014b. Genetic Variability and Sowing Dates Effect of Cluster Bean (*Cyamopsis tetragonoloba* L., Taub) Genotypes in Semi Arid Region of Maharashtra, India. *Plant Archiv.,* **14(1):** 1 – 6.

27. Pavlicek, A., Hrda, S. and Flegr, J. 1999. Free Tree: Freeware Program for Construction of Phylogenetic Trees on the Basis of Distance Data and Bootstrapping/Jackknife Analysis of the Tree Robustness. Application in the RAPD Analysis of the Genus *Frenkelia*. *Folia Biol. (Praha.),* **45**: 97-99.

28. Peakall, R. and Smouse, P. E. 2012. GenAlEx 6.5: Genetic Analysis in Excel. Population

Genetic Software for Teaching and Research: An Update. *Bioinforma.,* **28**: 2537 - 2539.

29. Pritchard, J. K., Stephens, M. and Donnelly, P. 2000. Inference of Population Structure Using Multi Locus Genotype Data. *Genet.,* **155**: 945 - 959.

30. Punia, A., Arora, P., Yadav, R. and Chaudhury, A. 2009a. Optimization and Inference of PCR Conditions for Genetic Variability Studies of Commercially Important Cluster Bean Varieties by RAPD Analysis. *Asia Pacific J. Mol. Biol. Biotechnol.*, **17**: 33 - 38.

31. Punia, A., Yadav, R., Arora, P. and Chaudhury, A. 2009b. Molecular and Morpho-physiological Characterization of Superior Cluster Bean (*Cymopsis tetragonoloba*) Varieties. *J. Crop Sci. Biotechnol.,* **12**: 143-148.

32. Saini, R. K., Saad, K. R., Ravishankar, G. A., Giridhar, P. and Shetty, N. P. 2013. Genetic Diversity of Commercially Grown *Moringa oleifera* Lam. Cultivars from India by RAPD, ISSR and Cytochrome P450-based Markers. *Plant Syst. Evol.,* **299**: 1205-1213.

33. Sharma, P., Sharma, V. and Kumar, V. 2014. Genetic Diversity Analysis of Cluster Bean [*Cyamopsis tetragonoloba* (L.) Taub] Genotypes Using RAPD and ISSR Markers. *J. Agri. Sci. Tech.,* **16**: 433 - 443.

34. Sharma, V., Sharma, T. R., Rana, J. C. and Chahota, R. C. 2015. Analysis of Genetic Diversity and Population Structure in Horse Gram (*Macrotyloma uniflorum*) Using RAPD and ISSR Markers. *Agr. Res.*, DOI: 10.1007/s40003-015-0165-7.

35. Singh, A., Dikshit, H. K., Jain, N., Singh, D. and Yadav, R. N. 2014. Efficiency of SSR, ISSR and RAPD Markers in Molecular Characterization of Mung Bean and Other *Vigna* Species. *Indian J. Biotechnol.*, **13**: 81-88.

36. Singh, P. K., Sharma, H., Srivastava, N. and Bhagyawant, S. S. 2014. Analysis of Genetic Diversity among Wild and Cultivated Chickpea Genotypes Employing ISSR and RAPD Markers. *Am. J. Plant Sci.*, **5**: 7.

37. Thompson, M. G. and Murray, W. F. 1980. Rapid Isolation of High Molecular Weight Plant DNA. *Nucleic Acid. Res.,* **8**: 4321-4325.

38. Vaishali, Khan S., Singh, N. P. and Sharma, V. 2014. Genetic Diversity Analysis of *Butea monosperma* from Different Agro-ecological Regions of India Using ISSR Marker System and Its Comparison with RAPD. *Euro. J. Biotechnol. Biosci.,* **2**: 32 - 36.

39. Welsh, J. and McClelland, M. 1990. Fingerprinting Genomes Using PCR with Arbitrary Primers. *Nucleic Acids Res.* **18**, 7213 - 7218.

40. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA Polymorphism Amplified by Arbitrary Primers

Are Useful as Genetic Markers. *Nucleic Acid. Res.,* **18**: 6531- 6535.

41. Yadav, K., Yadav, S. K., Yadav, A., Pandey, V. P. and Dwivedi, U. N. 2014. Comparative Analysis of Genetic Diversity among Cultivated Pigeonpea (*Cajanus cajan* (L) Millsp.) and Its Wild Relatives (*C. albicans* and *C. lineatus*) Using Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) Fingerprinting. *Am. J. Plant Sci.,* **5**: 1665 - 1678.

42. Yahia, Y., Hannachi, H. and Ferchichi, A. 2014. Genetic Diversity of *Vicia faba* L. Based on

Random Amplified Polymorphic DNA and Simple Sequence Repeat Markers. *Acta Botanica Gallica Bot. Lett.,* **161**: 151-158.

43. Zhao, F., Nie, J., Chen, M. and Wu, G. 2015. Assessment of Genetic Characteristics of *Aconitum* Germplasm in Xinjiang Province (China) by RAPD and ISSR Markers. *Biotech. Biotechnol. Equip.,* **29**: 309 - 314.

44. Zietkiewicz, E., Rafalski, A. J. and Labuda. D. 1994. Genome Fingerprinting by Simple Sequence Repeat (SSR): Anchored Polymerase Chain Reaction Amplification. *Genom.,* **20**: 176 - 183.

# تنوع ژنتیکی ژرم‌پلاسم لوبیا خوشه‌ای (*Cyamopsistetragonoloba* L.,Taub): محصولی با اهمیت غذایی و صنعتی

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## چکیده

به منظور اطلاع از میزان تنوع ژنتیکی درون گروهی توده‌های یک ژرم‌پلاسم، تنوع ژنتیکی ژنوتیپ‌های لوبیا خوشه‌ای (*Cyamopsistetragonoloba* L.; Fabaceae) با استفاده از نشانگرهای ISSR و RAPD مورد مطالعه قرار گرفت. استفاده از دو روش مطالعه داده‌ها به صورت منفک و توأم، گستره‌ای از تنوع ژنتیکی بین مجموع ۱۰۴ ژنوتیپ جمع‌آوری شده از مناطق مختلف جغرافیایی هند، نشان داد. روش خوشه‌بندی شبیه‌سازی شده، ژنوتیپ‌های گیاهی جمع‌آوری شده را به گروه‌های خویشاوند مجزا تفکیک نمود. استفاده از برنامه Structure و تجزیه خوشه‌ای داده‌های حاصل از نشانگرهای مولکولی منجر به ایجاد شش زیرگروه بزرگ و مجزا شد. این نتایج با استفاده از تجزیه به مؤلفه‌های اصلی نیز مورد تأیید قرار گرفت. استفاده از مجموع داده‌ها در مقایسه با تجزیه داده‌ها به صورت مجزا، اطلاعات مفیدتری ارائه کرد. در این مطالعه بازه تنوع ژنتیکی مشاهده شده گسترده بود و ایجاد شش خوشه بزرگ، نشان دهنده وجود اجداد ژنتیکی زیادی می‌باشد که می‌تواند در استفاده از تنوع ژرم‌پلاسمی موجود و به منظور انتخاب و اصلاح گیاه لوبیا خوشه‌ای، نقطه شروع سودمندی باشد.