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# Molecular diversity of chickpea (*Cicer arietinum* L.) genotypes differing in their Raffinose family Oligosaccharides viz., raffinose and stachyose content as revealed through SSR markers

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**Abstract**

From a nutritional point of view, the α -galactosides are believed to be implicated in the development of flatulence following the ingestion of legume seeds. One important group of these compounds is the soluble α -galactosides, all of which are characterized by the presence of α (1–6) links between the galactose molecules which are responsible for causing flatulence viz., raffinose family oligosaccharides (RFOs) like raffinose and stachyose. There is very little information about the genetic variability and diversity among the cultivated chickpea for RFOs. Diversity among 50 chickpea (*Cicer arietinum* L.) genotypes differing in their RFOs content was studied using SSR markers. Out of 86 primers tested, 36 reported polymorphism amplifying 81 alleles. Dendrogram based on Jaccard's similarity coefficients were generated based on an average linkage algorithm (UPGMA) using marker data. Genotypes were grouped into three clusters based on genetic distances and the UPGMA grouping could clearly discriminate the genotypes effectively as per their pedigree and origin. The grouping pattern also seems to have followed the pattern of RFOs content, seed type, seed size apart from the breeding centre from where they were developed. The present molecular diversity among the genotypes studied can be exploited effectively by crossing the genotypes of Cluster I and III with that of Cluster II as these both clusters are most further apart so as to obtain transgressive segregants for RFOs content and selection can be implemented for selecting lines with lower RFOs content. ICRISAT lines which were desi types had the highest RFOs content i.e. raffinose and stachyose and formed a distinct group (III). Similarly the kabuli breeding lines obtained from ICARDA, Syria also formed a distinct group (I) and had greater RFOs content compared to the breeding lines obtained from IARI. The lowest raffinose and stachyose was recorded in lines obtained from IARI, Chickpea Program lines.

**Keywords:** *Cicer arietinum* L., Grouping pattern, Genetic diversity, RFOs, SSR markers.
**Abbreviations**: IARI_Indian Agricultural Research Institute, ICARDA_International Center for Agricultural Research in the Dry Areas, ICRISAT_International Crops Research Institute for the Semi-Arid-Tropics, RFOs_Raffinose Family Oligosaccharides, UPGMA_Unweighted Pair Group Method with Arithmetic Mean, PCoA_Principal Component Analysis.

## Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important pulse crops in India. Chickpea has been divided into two broad groups as microsperma and macrosperma types based on seed size and as desi and kabuli based on seed shape. The Kabuli types are generally grown in the Mediterranean region including Southern Europe, Western Asia and Northern Africa while the desi types are grown mainly in Ethiopia and the Indian subcontinent. Out of the total world area of 11.97 million hectares and production of 10.89 million tons, India is the largest chickpea producing country with a share of about 68% in the global chickpea production (FAOSTAT, 2012) with 6.67 million hectares, 5.3 million tons which represents 30% and 38% of the national pulse acreage and production, respectively. Major chickpea producing states sharing over 95% area are Maharashtra, Gujarat, Andhra Pradesh, Karnataka, Madhya Pradesh, Uttar Pradesh and Rajasthan. Over several decades despite intensive breeding efforts, the average global chickpea yield of 0.9 t/ha (FAOSTAT, 2009) is far below its yield potential of 5 t/ha because of limited genetic variation present in germplasm. Conventional breeding approaches have not greatly improved yield. Genetic diversity, relationship knowledge and management within and between the cultivated chickpea and its wild relatives are of paramount importance and may ensure the long-term success of chickpea improvement programs. Chickpea breeders throughout the world are focusing on increasing yield by analysing diversity and identifying genes for resistance/tolerance into elite germplasm. Molecular markers have been shown to play a crucial role in crop improvement programs. Such markers serve as efficient and powerful tools for marker-assisted selection of agronomically important traits. Molecular marker technologies help in improving the efficiency of breeding several-fold since selection is not directly on the trait of interest but on the molecular marker tightly linked to the trait, thereby accelerating the generation of new varieties, especially when the characters are difficult to score. In addition to these applications, cultivated chickpea has low level of genetic polymorphism. However, now availability of large number of microsatellite markers is offering immense

scope in assessing the diversity and of utilizing the diverse lines in map construction. They also provide new insights into genome analysis, help in germplasm characterization, phylogenetic analysis and genetic diagnostics. It is important to characterize the genetic diversity in plant species since they serve as a resource base for yet unidentified genetic information (Bharadwaj et al., 2010). Germplasm collections needs to be analyzed using for estimating the genetic variability. Interest in the low molecular weight carbohydrates found in natural products has increased considerably during the past few years. One important group of these compounds is the soluble α -galactosides, all of which are characterized by the presence of α (1–6) links between the galactose moleculaes which are responsible for causing flatulence viz., galactosyl cyclitols like ciceritol, as well as Raffinose Family Oligosaccharides (RFOs) like raffinose and stachyose. From a nutritional point of view, the α -galactosides are believed to be implicated in the development of flatulence following the ingestion of legume seeds. The absence of α-galactosidase enzyme leads to undigested RFOs to reach the large intestine in humans (Rackis, 1975; Cristofaro et al., 1974) where the microflora anaerobically hydrolyse them to produce carbon dioxide, hydrogen, and methane gases, causing flatulence. (Kurbel et al., 2006; Price et al., 1988). This problem is considered to be the single most important factor that deters people from eating more grain legumes. The negative effect of RFOs in chickpea was reported by Martinez-Villaluenga et al. (2008) and Zia Ul Haq et al. (2007). Fleming (1981) studied the relationship between flatus Potential and carbohydrate distribution in legume seeds and indicated the role of RFOs in causing flatulence. Analysis of variance revealed a significant impact of genotype, environment and their interaction on RFOs content (Gangola et al., 2013). Cooking method was found to influence the RFOs content as well as nutritional properties in the final product and microwave cooking was found to significantly reduce the flatulence factors (Viveros et al., 2001; Saleh et al., 2006) There is very little information about the genetic variability and molecular diversity among the cultivated chickpea for RFOs. Considering the above, an investigation was planned for molecular characterization of chickpea genotypes differing for their raffinose and stachyose content using SSR primers

## Results

### *Molecular diversity*

86 SSR primer pairs amplified 1–3 loci per primer pair. Out of these, 36 SSR primer pairs were polymorphic while 7 were monomorphic. 81 amplicons were generated with an average 2.25 amplicons per primer pair. A high degree of molecular polymorphism was exhibited by all the markers studied. The polymorphic information content ranged from 0.29 to 0.99. The Jacard's similarity matrix dendrogram constructed using the UPGMA method showed the genetic similarity between lines ranged from 0.23 to 0.60. This study revealed that all the 50 varieties grouped into 3 major clusters with four sub-groups for the first and second group and three for the last remaining group (Fig 1). A critical examination of these 3 clusters clearly indicate that the grouping was primarily based on centre of origin with all those lines developed from Indian sub-continent (both from ICRISAT and various Indian Chickpea Breeding Centres) forming into distinct subgroups II and III. All those lines from West Asia and North Africa obtained mostly from ICARDA, Syria grouped into a distinct cluster. The primary grouping appears to follow geographic distribution from where these lines were obtained i.e. source or more precisely the origin of cultivars. All the lines which were from Central Asia grouped as one major cluster while those from India sub continent grouped as two major cluster distinct from cluster I (Syrian group). Both the arms in the tree between these two major groups are quite diverse indicating large variability at molecular levels between the Syrian group and the Indian group. There was a tie between the genotypes ICCV 05112 and ICCV 06108. This was obvious as both the lines had the same pedigree ie ICCV 2 x PDG 84-16. Similarly PG 0515 and Pusa 5023 which are extra bold seeded kabuli types seem to have grouped into cluster I which had only kabuli large seeded collections. PG 0515 is a local market selection from Rahuri India. It is quite well known that extra large seeded kabuli types were grown as 'dollar *chana*' in India are mostly introduced materials. Pusa 5023, a newly released extra large seeded kabuli type has FLIP 90-166 as one of the parents. The grouping had clear cut differences for RFOs content. All the genotypes in cluster I were extra large seeded kabuli types with higher RFOs content with raffinose, stachyose means of 12.64 % and 33.33 % respectively. Cluster II genotypes comprising mostly of those developed from IARI, New Delhi had raffinose of 10.01 % and stachyose of 30.34 %. The cluster III were desi types with very high RFOs content and had raffinose of 17.17 % and stachyose of 41.45 %. Simple leaf mutant bold desi type mutant 77 (SBD 77) and FLIP 90-166 were extensively used as donors for larger seed size at IARI centre in chickpea breeding program and thus these types appear to have grouped with cluster II. This clearly brings out the distinctiveness of the Mediterranean group of lines from Syria and vicinity to be distinct from the Indian sub continent lines. It is obvious as such because the ICRISAT germplasm has more than 60 % accession from Indian sub continent and use of these accessions in developing advance breeding lines and varieties would have narrowed the genetic base at this Centre. Further adaptive selection from these lines by breeders in India while in tier utilization, though they may involve wider crosses would have occurred due to selection drifts in generation advancement thus developing lines with a narrow base albeit suitable for cultivation to the climatic requirements of the Indian sub-continent. The grouping of lines from Syria farther away indicates the more diversity available in these lines as well as their vicinity to centre of diversity of chickpea which is the Mediterranean centre where in the entire wild forms also occur naturally. Thus a greater introgression of the lines from wild type would have occurred in this area. Results from the present study support the observations of several workers about the potential utility of molecular markers in characterization (Bharadwaj et al., 2010). There was reasonably high rate of polymorphism which points towards the scope for further utilization of these markers for chickpea improvement. The occurrence of unique alleles or rare SSR alleles provides an immense opportunity for generation of comprehensive fingerprint database. The resources of many unique SSR alleles may be an indication of addition or deletion of small number of repeats (Goldstein and Pollock, 1997) and most rational explanation for high mutation rate is polymerase slippage (Levinson and Gutman, 1987). This inherent character may also have contributed to the grouping of the genotypes as during meiotic recombination such addition or deletion of units has taken place. The PIC value is influenced by the occurrence of variants per locus as well as relative distribution of the alleles. The reasons for occurrence of two bands deviating from the expected single band for a homozygous genotype could be due to mutations that could have occurred in the

**Table 1.** Genotypes and their parentage along with the RFOs content used for studying genetic and quality diversity.

| Sl no. | Genotypes | Parentage | Seed type |
|---|---|---|---|
| 1. | ILC 464 | ACC no 26595-68 | Kabuli |
| 2. | ILC 2555 | ICC 7589 | Kabuli |
| 3. | FLIP 81-71C | X79 TH151/ILC 72 x ILC 897 | Kabuli |
| 4. | FLIP 83-7C | X80 TH264/(ILC 480 x ILC 72) x ILC 263 | Kabuli |
| 5. | FLIP 84-48C | X81 TH55/ILC 1920 x ILC 2956 | Kabuli |
| 6. | FLIP 84-79C | X80 TH176/ILC 72 x ILC 215 | Kabuli |
| 7. | FLIP 84-188C | X81 TH48/ILC 1920 x ILC 201 | Kabuli |
| 8. | FLIP 85-1C | X82 TH60/ILC 95 x ILC 2956 | Kabuli |
| 9. | FLIP 85-17C | X83 TH19/FLIP82-65C x FLIP82-69C | Kabuli |
| 10. | FLIP 86-5C | X81 TH199(ILC 202(WH) x ILC 3355 | Kabuli |
| 11. | FLIP 86-6C | X81 TH203(ILC 3279(WH) x ILC 3355 | Kabuli |
| 12. | FLIP 87-8C | X85 TH246/ILC 3398 x FLIP 83-13C | Kabuli |
| 13. | FLIP 97-137C | X94TH12/FLIP90-132CXS91347 | Kabuli |
| 14. | FLIP 97-263C | X94TH71/FLIP87-59CXUC 15 | Kabuli |
| 15. | FLIP 97-266C | X94TH75/FLIP87-58CXUC 15 | Kabuli |
| 16. | FLIP 97-281C | X94TH75/FLIP87-58CXUC 15 | Kabuli |
| 17. | FLIP 97-503C | X94TH8/FLIP86-6CXFLIP90-109C | Kabuli |
| 18. | FLIP 97-530C | X94TH103/(FLIP91-186CXFLIP91-96C)XFLIP90-109C | Kabuli |
| 19. | FLIP 97-706C | X94TH114/(FLIP91-138CXFLIP85-60C)XFLIP91-133C | Kabuli |
| 20. | FLIP 98-121C | X95TH 42 /(FLIP90-15CXILC5362)XFLIP93-2C | Kabuli |
| 21. | Pusa 1053 | ICCV 3 x Flip 88-120 | Kabuli |
| 22. | Pusa 5023 | (Flip 90-166 x BG 1072)x(BG 1082 x BG 1073) | Kabuli |
| 23. | Pusa 2024 | (BG 261 x ICC 88503) x (GL 920 x BG 1003) | Kabuli |
| 24. | Pusa 1088 | (Pusa 256 x ICCV 32) x ICCV 32 | Kabuli |
| 25. | Pusa 1108 | (BG 315 x ILC 72) x (ICC 13 x Flip) x (ICCV 32 x Surutoto 77) | Kabuli |
| 26. | PG 0515 | Kabuli extra-large seeded local selection material from Rahuri, Maharastra | Kabuli |
| 27. | FLIP 90-166 | Kabuli extra-large seeded breeding line from ICARDA | Kabuli |
| 28. | Pusa 1105 | (C 104 x BG 1003) x (ICC 88503 x BG 1048) | Kabuli |
| 29. | Pusa 1003 | ICCV 32 x Rabat | Kabuli |
| 30. | Pusa 391 | ICC 3935 x Pusa 256 | Desi |
| 31. | Pusa 372 | P1231 x P1265 | Desi |
| 32. | Pusa 362 | (BG 203 x P 179) x BC 203 | Desi |
| 33. | Pusa 256 | (JG62x 850-3/27) x (L550 x H 208) | Desi |
| 34. | Pusa 72 | (Pusa 256 x E 100 YM) x Pusa 256 | Desi |
| 35. | Pusa 1103 | (Pusa256 x *C.reticulatum*) x Pusa 362 | Desi |
| 36. | BGD 112 | (BG 209 x GL 84038) x Pusa 212 | Desi |
| 37. | SBD 377 | ICCV 88109 x PRR 1) x ICC 4958 | Desi |
| 38. | Pusa 5028 | (SBD 377x Pusa 362) x (SBD 377x BGD 72) | Desi |
| 39. | ICCV 00104 | JG 74 x ICCL 83105 | Desi |
| 40. | ICCV 03102 | (ICCV 92014 x JG 23) x BG 1032 | Desi |
| 41. | ICCV 03103 | (ICCV 92014 x JG 23) x BG 1032 | Desi |
| 42. | ICCV 03210 | (ICCV 92014 x JG 23) x BG 1032 | Desi |
| 43. | ICCV 03211 | (ICCV 92014 x JG 23) x BG 1032 | Desi |
| 44. | ICCV 04110 | (ICCV 89224 x JG 11) x BG 390 | Desi |
| 45. | ICCV 04111 | (ICCV 93001 x JAKI 9218) x BG 256 | Desi |
| 46. | ICCV 05112 | ICCV 2 x PDG 84-16 | Desi |
| 47. | ICCV 06108 | ICCV 2 x PDG 84-16 | Desi |
| 48. | ICCV 06109 | ICC 4958 x ICCV 97303 | Desi |
| 49. | ICCV 97022 | ICCL 84226 x ICCL 86103 | Desi |
| 50. | ICCV 97114 | ICCV 10 x K 850 | Desi |

parental stock at a specific SSR locus, leading to two different alleles on homologous chromosomes. Another possible cause of variation in the germplasm lines is the presence of active mobile elements.

***PCoA analysis***

The grouping results were in conformity with the PCoA results of the dendrogram. The two dimensional PCoA plot (Fig 2) separated all the accessions into two major clusters. All the accessions developed from IARI appeared at the top of the x- axis as a separate major cluster whereas the majority of the accessions from the ICARDA centre appeared at the right bottom. The accessions from ICRISAT also formed a distinct cluster at the left bottom of the X-axis.

**Discussion**

A thorough utilization of genetics resources is only possible if the amount of diversity and the genetic relationships of the collection are known, A narrow genetic base in chickpea (Nguyen et al., 2004; Singh et al., 2003) urgently warrants base broadening efforts. Thus the need to investigate the genetic diversity among the germplasm collections is there. Investigations into nature and structure of genetic diversity gives an idea of relatedness as well as ability to identify germplasm sources having valuable genes for yield, quality

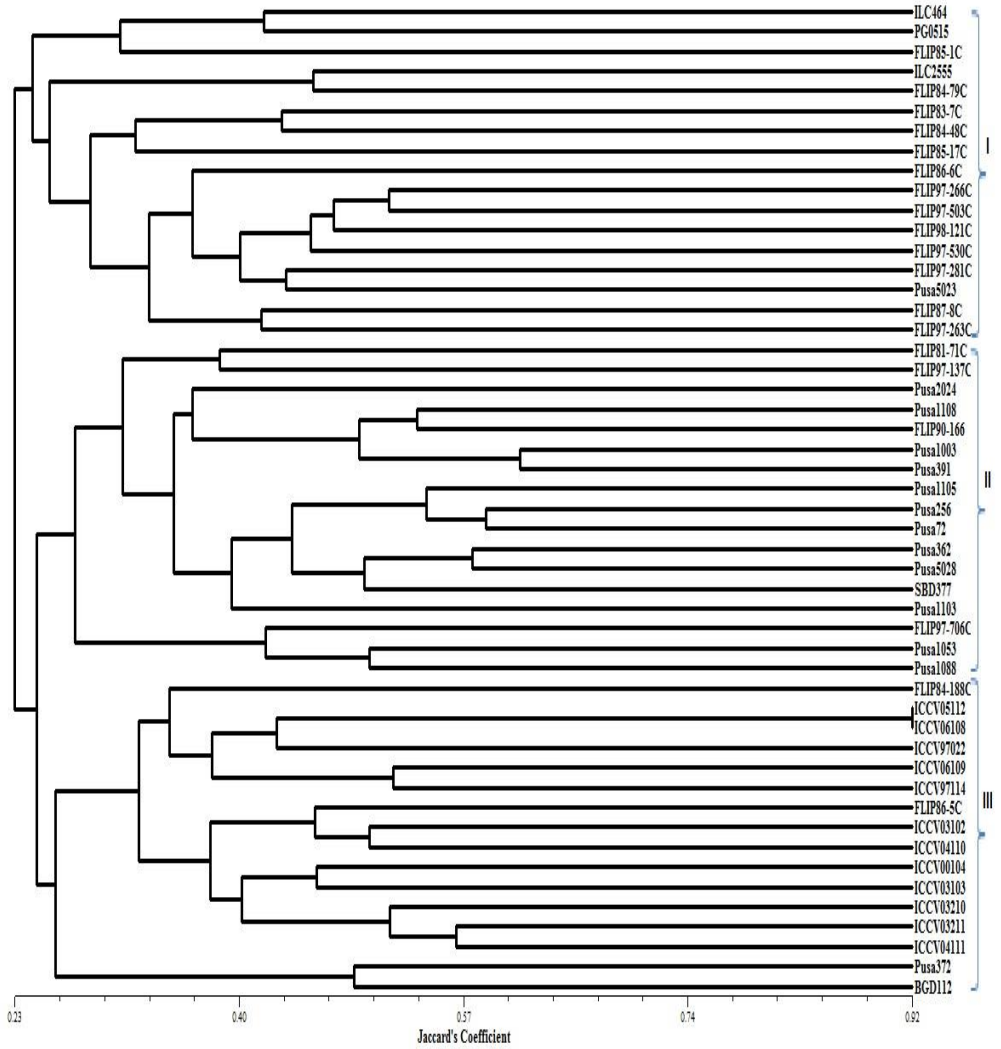


**Fig 1.** Dendrogram showing Jaccard's dissimilarity produced using UPGMA cluster analysis demonstrating association among 50 genotypes of chickpea.

and other important traits (Choudhary et al., 2012) SSR markers have been now widely used in chickpea for studying diversity. Research efforts have contributed largely to the increase in the SSR markers available for characterizing diversity. For instance ca. 2000 SSR markers have been developed from genomic DNA libraries (Nayak et al., 2010; Gaur et al., 2011), ESTs (Varshney et al., 2009) and BAC-end sequences (Thudi et al., 2011). In this study, we evaluated 86 SSR markers in 50 chickpea accessions representing popular cultivated chickpea cultivars and breeding lines. The SSR analysis showed considerable genetic diversity. 81 amplicons were generated with an average 2.25 amplicons per primer pair. The ability of SSRs to detect intra- as well as interspecific variation in chickpea has been demonstrated previously. For instance, Huttel et al. (1999) detected 2 to 4 alleles at the intraspecific level in four genotypes using 22 SSR markers. Studying 2915 genotypes with 48 SSRs, Upadhyaya et al. (2008) reported an average of 35 alleles per locus with PIC of 0.85. The higher allele number detected in the present study compared to the studies of Huttel et al. (1999) and Singh et al. (2008) can be attributed to the use of a larger set of microsatellite markers. On the other hand, lower values of alleles and PIC relative to Upadhyaya et al. (2008) are due to the use of a much smaller germplasm set (2%). There was a lower proportion of heterozygous alleles in the present study. Heterozygosity in self- pollinated crops like chickpea arises due to very low level of out crossing (Gowda, 1981) or due to other possibilities like inbreeding depression at the loci in question or a higher mutation rate, the presence of heterozygotes cannot be completely ruled out in an otherwise self-pollinated crop. The occurrence of distinct groups of chickpea lines revealed through SSR analysis could possibly draw the attention of the chickpea breeders for effective pre-breeding for breaking yield barriers. The initial gains obtained through use of germplasm from ICRISAT though has paid off, wider gains and introgression of alleles for more useful traits can occur if the pre-breeding involves ICARDA germplasm or wild relatives. Importance of wild relatives of chickpea and pre-breeding as a source of genes having resistance to biotic and abiotic traits has been documented by many chickpea workers (Singh 2005; Kaur et al., 2010). Thus, molecular characterization can give very useful information to chickpea breeder. The ability to discern genetic variation among the wild accessions to that of cultivated types and within the cultivated types depending on the geographic origin was clearly brought forward by the use of SSR markers in the present study.

**Table 2.** Mean performance of 50 chickpea genotypes evaluated for Raffinose and Stachyose content in seeds.

| Genotypes | Raffinose content | Stachyose content |
|---|---|---|
| ILC 464 | 11.71±0.28 | 20.60±0.35 |
| ILC 2555 | 18.76±0.56 | 23.41±0.28 |
| FLIP 81-71C | 16.34±0.57 | 24.62±0.40 |
| FLIP 83-7C | 10.30±0.58 | 41.07±0.57 |
| FLIP 84-48C | 12.20±0.37 | 39.09±0.37 |
| FLIP 84-79C | 14.69±0.34 | 38.46±0.30 |
| FLIP 84-188C | 16.42±0.59 | 37.69±0.32 |
| FLIP 85-1C | 18.95±0.23 | 35.96±0.51 |
| FLIP 85-17C | 10.17±0.59 | 24.68±0.08 |
| FLIP 86-5C | 10.67±0.30 | 40.96±0.34 |
| FLIP 86-6C | 11.29±0.51 | 40.14±0.13 |
| FLIP 87-8C | 12.65±0.54 | 38.51±0.65 |
| FLIP 97-137C | 14.74±0.31 | 37.48±0.63 |
| FLIP 97-263C | 15.47±0.55 | 36.26±0.28 |
| FLIP 97-266C | 15.89±0.72 | 25.23±0.41 |
| FLIP 97-281C | 9.54±0.43 | 41.60±0.56 |
| FLIP 97-503C | 11.73±0.38 | 42.53±0.38 |
| FLIP 97-530C | 13.63±0.37 | 43.82±0.13 |
| FLIP 97-706C | 13.61±0.93 | 44.76±0.61 |
| FLIP 98-121C | 12.73±0.35 | 46.10±0.24 |
| Pusa 1053 | 7.55±0.28 | 30.35±0.35 |
| Pusa 5023 | 6.81±0.22 | 14.58±0.84 |
| Pusa 2024 | 7.13±0.23 | 39.50±0.48 |
| Pusa 1088 | 4.36±0.17 | 19.50±0.52 |
| Pusa 1108 | 14.43±0.28 | 35.50±0.53 |
| PG 0515 | 8.46±0.40 | 14.49±0.53 |
| FLIP 90-166 | 8.91±0.16 | 20.47±0.62 |
| Pusa 1105 | 9.57±0.22 | 33.42±050 |
| Pusa 1003 | 7.54±0.48 | 34.42±0.22 |
| Pusa 391 | 9.55±0.44 | 42.65±0.43 |
| Pusa 372 | 7.70±0.24 | 36.44±0.59 |
| Pusa 362 | 14.73±0.38 | 39.73±0.33 |
| Pusa 256 | 13.28±0.35 | 32.65±0.82 |
| Pusa 72 | 9.73±0.23 | 24.02±0.30 |
| Pusa 1103 | 2.94±0.52 | 7.44±0.26 |
| BGD 112 | 8.75±0.59 | 17.88±0.48 |
| SBD 377 | 8.66±0.37 | 13.21±0.19 |
| Pusa 5028 | 8.42±0.04 | 36.07±0.44 |
| ICCV 00104 | 19.63±0.22 | 48.24±0.33 |
| ICCV 03102 | 26.04±0.67 | 53.96±1.33 |
| ICCV 03103 | 16.99±0.16 | 45.37±0.35 |
| ICCV 03210 | 23.14±0.35 | 51.24±0.28 |
| ICCV 03211 | 16.21±0.16 | 43.24±0.47 |
| ICCV 04110 | 15.01±0.17 | 37.69±0.24 |
| ICCV 04111 | 19.10±0.26 | 40.85±0.35 |
| ICCV 05112 | 22.00±0.75 | 44.54±0.69 |
| ICCV 06108 | 25.34±0.38 | 47.50±0.29 |
| ICCV 06109 | 16.92±0.33 | 43.45±0.24 |
| ICCV 97022 | 13.77±0.38 | 35.53±0.49 |
| ICCV 97114 | 17.08±0.44 | 38.70±0.59 |
| Mean | 13.22 | 34.91 |
| Range | 2.94-26.04 | 7.44-53.96 |
| SE | 0.68 | 0.83 |
| LSD 5% | 1.12 | 1.35 |
| CV | 5.20 | 2.38 |

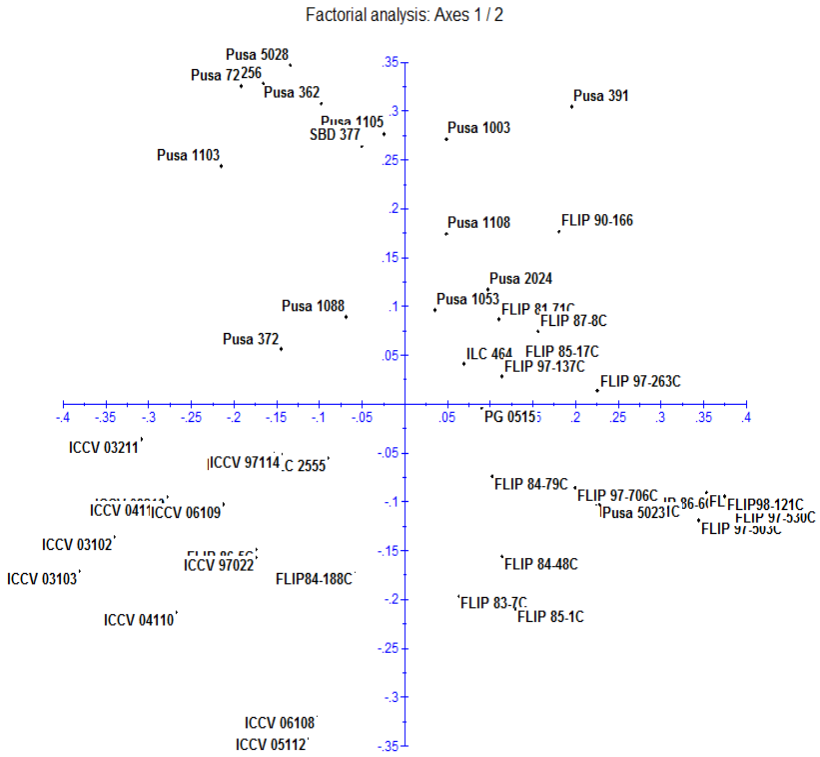


**Fig 2.** PCoA of 50 chickpea genotypes using 36 SSR markers.

***Diversity vis-à-vis RFOs content***

Chickpea is an excellent source of protein. The presence of anti-nutritional factors like the raffinose family oligosachharides (RFOs) restrict its use and acceptability as a food (Olmedilla Alonso et al., 2010). The presence of some bioactive substances like enzyme inhibitors, phenolic compounds, raffinose oligosaccharides etc., chickpea consumption is limited. The RFOs are non-digestive oligosaccharides and are considered as anti-nutrients since they are thought to be the major producers of flatulence due to the absence of α-galactosidases enzyme in the human intestine, consequently undergoing bacterial fermentation. These oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria with gas expulsion (H2, CO2, and traces of CH4), causing the flatus effect and sometimes diarrhea and abdominal pain and a factor which has tended to render it less acceptable. This problem is considered to be the single most important factor that deters people from eating more grain legumes. There is very little information about the genetic variability and diversity among the cultivated chickpea for RFOs. Understanding the extent of natural variation of RFOs among cultivated chickpea at molecular level is essential to develop pre-breeding and breeding strategies for chickpea. The narrow genetic base among cultivated chickpea accessions is limiting genetic improvement of chickpea through breeding efforts. These are generally present in the chickpea seeds. RFOs accumulate in seeds and play an important physiological role in the plants (Martinez – Villaleunga et al., 2008). They are important in seed longevity, (Koster, 1991), free radical scavengers (Bolouri-Moghaddam et al 2010) and tolerance to stresses (Cho et al., 2010). However they affect human health negatively. Chickpea contains high quantities of RFOs and galactosyl cyclitols. The concentrations of raffinose, stachyose and verbascose in chickpeas are about 1.5 g, 2.6 g and 0.2 g 100 g-1 dry matter, respectively (Alajaji and El-Adawy, 2006). A study of three chickpea cultivars found the raffinose concentration to range from 1.9 - 2.8 g 100 g-1 dry matter, whereas the combined stachyose plus verbascose concentration was 0.9 - 1.7 g 100 g-1 dry matter (Mulimani and Ramalingam 1997). Chickpea improvement for reduction in RFOs content requires natural variation to be present for this trait. An extensive evaluation of variation and diversity of RFOs concentration in cultivated chickpea germplasm collections has not yet been carried out. Such assessment of variation in RFOs concentration and composition of oligosaccharides will not only help in the selection of genotypes with reduced RFOs concentration for chickpea seed quality improvement programs but will also provide material for a thorough understanding of RFOs biosynthesis in chickpea seeds. A large variation was seen in the RFOs content among the fifty genotypes studied. It was further observed that the genotypes obtained from ICRISAT and ICARDA had considerably larger RFOs content than those bred and released from IARI centre. A large variation in RFOs concentration along with starch and protein in a germplasm collection of 152 genotypes studied was also reported by Gangola et al (2012). Genotype and growing environment interaction also show a positive correlation between substrates of raffinose family oligosaccharides biosynthesis and their accumulation in chickpea (Gangola et al., 2013). These studies coupled with the present one indicate that here exists variation for this trait in the cultivated chickpea germplasm and selection can be exercised.The UPGMA dendrogram separated all the chickpea accessions into three major clusters, two (cluster II and III) representing accessions from Indian subcontinent

**Table 3** Annealing temperature, allele number, polymorphic information content (PIC), gene diversity and heterozygosity ($H_O$ %) obtained after screening 50 chickpea genotypes at 36 SSR loci.

| Sl.no | Primers | Sequence | Annealing temperature | Allele No | PIC | Gene Diversity | $H_o$ (%) |
|---|---|---|---|---|---|---|---|
| 1 | TR56 | (F) TTGATTCTCTCACGTGTAATTC | 48.4 | | | | |
| | | (R) ATTTTGATTACCGTTGTGGT | 48.4 | 2 | 0.352 | 0.456 | 0.021 |
| 2 | CAM1068 | (F) TGGATGCAAAAGATTTGAGC | 53.2 | | | | |
| | | (R) TTCAAAGAAAGAAACACTTTTTCAA | 53.1 | 2 | 0.348 | 0.449 | 0.000 |
| 3 | TA80 | (F) CGAATTTTTACATCCGTAATG | 49.3 | | | | |
| | | (R) AATCAATCCATTTTGCATTC | 48.9 | 2 | 0.362 | 0.474 | 0.000 |
| 4 | TA113 | (F) TGCAAAAACTATTACGTTAATACCA | 54.8 | | | | |
| | | (R) TTGTGTGTAATGGATTGAGTATCTCTT | 54.3 | 2 | 0.341 | 0.436 | 0.000 |
| 5 | TA53 | (F)GGAGAAAATGGTAGTTTAAAGAGTACTAA | 53.1 | | | | |
| | | (R) AAAAATATGAAGACTAACTTTGCATTTA | 53 | 2 | 0.371 | 0.492 | 0.625 |
| 6 | GA9 | (F) GAACGGATTGGATGAAGCAT | 53.6 | | | | |
| | | (R) GTGCAAACAACCCTTTTTGG | 54.3 | 3 | 0.233 | 0.249 | 0.000 |
| 7 | TR59 | (F) AAAAGGAACCTCAAGTGACA | 48.4 | | | | |
| | | (R) GAAAATGAGGGAGTGAGATG | 48.1 | 2 | 0.372 | 0.495 | 0.000 |
| 8 | TA170 | (F) TATAGAGTGAGAAGAAGCAAAGAGGAG | 55.1 | | | | |
| | | (R) TATTTGCATCAATGTTCTGTAGTGTTT | 55.2 | 2 | 0.374 | 0.498 | 0.000 |
| 9 | TA127 | (F) AAATTGTAAGACTCTCATTTTTCTTTATT | 52.8 | | | | |
| | | (R) TCAAATTAACTACATCATGTCACACAC | 53.9 | 2 | 0.195 | 0.219 | 0.000 |
| 10 | TA176 | (F) ATTTGGCTTAAACCCTCTTC | 49.3 | | | | |
| | | (R) TTTATGCTTCCTCTTCTTCG | 48.9 | 2 | 0.286 | 0.346 | 0.000 |
| 11 | TA108 | (F) AAACCATTATCGAGTTGGATATAAAGA | 54.9 | | | | |
| | | (R) TTTCTAAGTGTTCTTTTCTTAGAGTGTGA | 54.8 | 3 | 0.557 | 0.635 | 0.000 |
| 12 | TS29 | (F) AACATTCATGAACCTACCTCAACTTA | 53.5 | | | | |
| | | (R) CCATATGAGTACACTACCTCTCGG | 54.3 | 3 | 0.445 | 0.538 | 0.000 |
| 13 | TA21 | (F) GTACCTCGAAGATGTAGCCGATA | 54.1 | | | | |
| | | (R) TTTTCCATTTAGAGTAGGATCTTCTTG | 54.8 | 1 | 0.000 | 0.000 | 0.000 |
| 14 | TAA137 | (F) CATGATTTCCAACTAAATCTTGAAAGT | 61 | | | | |
| | | (R) TCTTGTTTCGTTTAAACAATTTCTTCT | 54.5 | 2 | 0.362 | 0.475 | 0.000 |
| 15 | CaSTMS15 | (F) CTTGTGAATTCATATTTACTTATAGAT | 47.2 | | | | |
| | | (R) ATCCGTAATTTAAGGTAGGTTAAAATA | 52.1 | 3 | 0.468 | 0.564 | 0.000 |
| 16 | TA59 | (F) ATCTAAAGAGAAATCAAAATTGTCGAA | 55.1 | | | | |
| | | (R) GCAAATGTGAAGCATGTATAGATAAAG | 54.7 | 3 | 0.497 | 0.571 | 0.000 |
| 17 | TA18 | (F) AAAATAATCTCCACTTCACAAATTTTC | 54.6 | | | | |
| | | (R) ATAAGTGCGTTATTAGTTTGGTCTTGT | 55.1 | 3 | 0.572 | 0.645 | 0.000 |
| 18 | TAA55 | (F) GGAACAACAACAACTCAAATG | 49.3 | | | | |
| | | (R) TGCTATTAAGTGTGACCAGCAAA | 54.1 | 3 | 0.525 | 0.592 | 0.043 |
| 19 | NCPGR4 | (F) TTACAGCTTGTGCTCAG | 47.1 | | | | |
| | | (R) AGTCAGATTCTTATCCGA | 38.9 | 3 | 0.502 | 0.580 | 0.000 |
| 20 | TA76 | (F) TCCTCTTCTTCGATATCATCA | 48.6 | | | | |
| | | (R) CCATTCTATCTTTGGTGCTT | 48.3 | 2 | 0.374 | 0.498 | 0.310 |
| 21 | TR31 | (F) CTTAATCGCACATTTACTCTAAAATCA | 54.5 | | | | |
| | | (R) ATCCATTAAAACACGGTTACCTATAAT | 54.1 | 1 | 0.000 | 0.000 | 0.000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 22 | TR29 | (F) GCCCACTGAAAAATAAAAAG | 48.4 | | | | |
| | | (R) ATTTGAACCTCAAGTTCTCG | 48.2 | 1 | 0.000 | 0.000 | 0.000 |
| 23 | GA137 | (F) GGGGGAAGATATGTTGGGTT | 53.9 | | | | |
| | | (R) GATCCAACGGGAACAAAGAC | 53.1 | 2 | 0.253 | 0.298 | 0.000 |
| 24 | GA102 | (F) CAGAGAACCACATGTTTAGTTGAA | 54.1 | | | | |
| | | (R) AGTTTTGATGCGTGCCATTT | 54.4 | 1 | 0.000 | 0.000 | 0.000 |
| 25 | CaSTMS14 | (F) TTGTGTTTCTCCTAATATTCTATTAGC | 513 | | | | |
| | | (R) GAATATGAATAACGTTACA | 36.6 | 1 | 0.000 | 0.000 | 0.000 |
| 26 | TA71 | (F) CGATTTAACACAAAACACAAA | 48 | | | | |
| | | (R) CCTATCCATTGTCATCTCGT | 48.5 | 1 | 0.000 | 0.000 | 0.000 |
| 27 | TA3 | (F) AATCTCAAAATTCCCCAAAT | 49.3 | | | | |
| | | (R) ATCGAGGAGAGAAGAACCAT | 49 | 2 | 0.371 | 0.493 | 0.000 |
| 28 | CAM0317 | (F) TGGCCTAAATGTCTCAGCAA | 61 | | | | |
| | | (R) AGAGGCAAACAAGAACCGAA | 53 | 3 | 0.579 | 0.654 | 0.040 |
| 29 | CAM0443 | (F) TCGTTTGCATAAGATGGAACA | 61 | | | | |
| | | (R) GTACAACCGCCGCAAATATC | 53 | 3 | 0.579 | 0.654 | 0.000 |
| 30 | TA130 | (F) TCTTTCTTTGCTTCCAATGT | 48.8 | | | | |
| | | (R) GTAAATCCCACGAGAAATCAA | 50.7 | 2 | 0.375 | 0.500 | 0.000 |
| 31 | H2I10 | (F) CATTAATTTGGGATTTTGTTTCAA | 54 | | | | |
| | | (R) GCATCACATTATTTTGTTCTTGTG | 53 | 3 | 0.504 | 0.571 | 0.000 |
| 32 | H3H04 | (F) TGTTTCCTGATGTTGAGAAACTC | 52.1 | | | | |
| | | (R) TATTTTATGATATCCGCGGTGAC | 52.4 | 2 | 0.343 | 0.440 | 0.000 |
| 33 | HIF05 | (F) GAGAGAGAGGAAGGGAAACG | 53.5 | | | | |
| | | (R) TCCTAACTTGCTCCTTAACCTTG | 50 | 3 | 0.512 | 0.578 | 0.020 |
| 34 | TA47 | (F) TTTTTATAGGTGTCTTTTTGTTGTCTTT | 54.6 | | | | |
| | | (R)TCTGAATAGGAAATAAGAAAGGTAGGTT | 54.9 | 3 | 0.516 | 0.584 | 0.000 |
| 35 | HIF21 | (F) GTTTCGCTCACATACCATCG | 52.7 | | | | |
| | | (R) GGGAAAGTCTTGCTCCTACG | 52.8 | 3 | 0.485 | 0.569 | 0.000 |
| 36 | TA25 | (F) AGTTTAATTGGCTGGTTCTAAGATAAC | 54.1 | | | | |
| | | (R) AGGATGATCTTTAATAAATCAGAATGA | 53 | 3 | 0.515 | 0.595 | 0.000 |

mostly and the third (cluster I) representing the ICARDA lines. The PCoA displayed a similar profile of major clusters, with minor deviations. Bharadwaj et al. (2010) reported a similar profile, with clusters clearly discriminating the accessions of cultivated chickpeas from Indian subcontinent and ICARDA lines from West Asia and North Africa region.

From Table 2, it is quite evident that both the ICRISAT lines which were desi had the highest RFO content i.e. raffinose and stachyose viz., ICCV 03102 (26.04, 53.96) followed by ICCV 06108 (25.34, 47.5) and ICCV 03210 (23.14, 51.2) formed a distinct group (III) (Fig 1). Similarly the kabuli breeding lines obtained from ICARDA, Syria also formed a distinct group (I) and had greater RFOs content compared to the breeding lines obtained from IARI. The lowest raffinose and stachyose was recorded for Pusa 1103 (2.94, 7.44) followed by Pusa 1088 (4.36, 19.5). These lines were developed from IARI, Chickpea Program. The grouping pattern also seems to have followed the pattern of RFOs content and seed size apart from the breeding centre from where they were developed. The present molecular diversity among the genotypes studied can be exploited effectively by crossing the genotypes of Cluster I and III with that of Cluster II to obtain transgressive segregants for RFOs content and selection can be implemented for selecting lines with lower RFOs content. Since ICCV 03102 and Pusa 1103 are farthest apart, a best parental combination suggested to exploit such a strategy would be crossing ICCV 03102 with Pusa 1103.

## Materials and Methods

### *Plant materials*

The present study was carried out at the Experimental Farm and Laboratory, Division of Genetics, IARI, New Delhi during 2011-12. Fifty genotypes of kabuli and desi chickpea lines including varieties/ elite lines from different agro-climatic zones of India and few genotypes of exotic origin were taken for this study (Table 1).

### *Estimation of RFOs content*

Raffinose and stachyose content (Table 2) was estimated by HPLC chromatograms as described by Xiaoli X et al. (2008)Three samples, one each from each replication was used in the study.

### *DNA extraction*

Isolation of DNA was carried out using modified CTAB method as described by Murray and Thompson (1980) later modified by Doyle and Doyle (1990).

### *PCR amplification*

86 SSR loci were screened in the varieties of which only 36 were polymorphic (Table 3). Biorad MyCycler thermal cycler, USA was used to carry out amplifications in 10 µl volumes which had 20–25 ng plant genomic DNA, 10×Tris buffer (15 mM $MgCl_2$ and Gelatine) of Bangalore Genei, India, 10 mM dNTP mix, 1.0 µl primer and 0.3 µl of 3U/µl Taq (Bangalore Genei, India). PCR analysis was taken up by having preparation of 150 sec. at 90ºC, followed by 18 cycles of denaturation at 94ºC for 20 sec., annealing for 50 sec. at 50ºC (touch down of 0.5ºC for every repeat cycle) and 1 min. elongation at 72ºC for 50 sec. Further 20 cycles of denaturation at 94ºC for 20 sec., annealing for 50 sec. at 55ºC and 50 sec. elongation at 72ºC were given and finally extension at 72ºC for 7 min. were performed. The amplified fragments were resolved on 2 per cent agarose gel.

### *Data analysis*

Band patterns for each of the microsatellites markers were recorded for each genotype by assigning a letter to each band. Alleles were numbered as 'A1', 'A2' etc. sequentially from the largest to the smallest sized band. No distinction was made between amplified products of varied intensity, when the amplified products were within the expected size range. Any band thought to be an artifact or bands which were either diffused or highly faint or those that were difficult to score due to multiple bands were considered as 'missing data' and was not considered while analyzing the genetic similarities (Bharadwaj et al 2010). The polymorphic bands were scored in a spread sheet format with '0' representing absence of band and '1' representing the presence of band. 'Null allele' for any specific marker in any genotype was again considered as absence of band (designated as '0'). The null alleles were reconfirmed. Monomorphic data were excluded from the studies except in cases where at least one genotype showed a null allele, clearly indicating absence of SSR primer binding site. Any marker with more than or equal to 30% missing data across various genotype was excluded from the analysis. The data were analyzed in NTSYS-PC software (version 2.1b). Utilizing binary data generated by SSR primers Jaccard's similarity coefficients (Jaccard, 1908) were calculated between genotypic pairs using NTSYS-pc 2.02 programme (Rohlf, 1989). From the similarity coefficients matrix, thus generated, the dissimilarity coefficients (JD; Genetic distances =1- similarity coefficient) were calculated. For Clustering, UPGMA was used based on the similarity matrix generated on combined data. Using the data matrix for the presence and absence of each allele, a PCoA was performed using the same software and the two principal coordinates were used to visualise the dispersion of genotypes. The genetic diversity such as observed heterozygosity, gene diversity, number of alleles per locus and polymorphic information content were calculated for the 50 genotypes using POWERMARKER 3.25 software (Liu and Muse, 2005).

## Conclusion

Microsatellite genotypic data from a number of loci have potential to provide unique allelic profiles or DNA fingerprints for establishing genotypes identity as well as in development of molecular linkage map of chickpea. Microsatellite or SSR is a group of repetitive DNA sequences that represents significant portion of eukaryotic genomes. The uniqueness and value of SSR arises from their multiallelic nature, co-dominant transmission, relative abundance and extensive genome coverage. Being PCR based and easily reproducible has become favourite tools with breeders and biotechnologists to discern the traits as well as to study diversity among cultivars The present analysis gives an insight of the interrelationship among the genotypes and highlights the urgency for effective supplementation of morphological data with the database generated by SSR marker to efficiently unearth the genetic inter-relationship among the genotypes. The SSR markers tried could clearly differentiate the lower RFOs content lines to that of higher RFOs lines. The diversity of *C. arietinum* lines from Mediterranean region of those of ICARDA, Syria to those from the India sub-continent also indicates that

greater genetic gains can be obtained by using these lines in the crossing programs and these being cultivated chickpea, crossing is easier and wider variability in the segregating generations would provide the chickpea breeder with greater initial material for carrying out selections.

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