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ELUCIDATING GENETIC DIVERSITY AND ASSOCIATION MAPPING TO IDENTIFY SSR MARKERS LINKED TO YIELD TRAITS AND PROTEIN CONTENT IN CHICKPEA (*CICER ARIETINUM* L.)

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ABSTRACT

Chickpea has played a major role in realization of 'Pulse Revolution' in India making the country near self-sufficient in Pulses. Chickpeas are high in protein. Protein is a necessary component of life. Pulses have two to three times the protein content of cereals. It is a significant source of protein in the Indian diet. However, potentiality of the protein has not been fully exploited as a source of protein in human diets. Forty two genotypes inclusive of checks were taken for the study. Protein content was isolated from seed as well as from leaves to understand the translocation of protein from source (leaves) to sink (seeds). The chickpea genotypes were divided into two sub groups based on the result of Structure Harvester, as delta K kinship was highest at K=2. Seven chickpea SSR markers were found to be tightly linked with the days to maturity, primary branches, biological yield, plant height, height of first pod, seed yield per plant, secondary branches, seeds per pod, protein and hundred seed weight. These markers covered the linkage groups # 2, 3, 5 and 7. CaM1068 (LG#5) was the chickpea marker tightly associated with protein, hundred seed weight and seed yield per plant.

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INTRODUCTION

The developing countries share the maximum area under production and consumption of chickpea. It is mainly grown in SE Asian countries with cultural, religious and nutritional values. Two main types of cultivated chickpeas are Kabuli (white seeded) and desi (brown seeded), representing the two diverse gene pools (Nawroz and Hero, 2011). The agricultural practices and successive breeding has narrowed down the genetic base of cultivated chickpea (Robertson *et al.,* 1997). Chickpea among the legumes rank third worldwide (FAO, 2006) and protein content of chickpea cultivars ranges between 20 and 22 percent (Pundir *et al.* 1988 and Jadhav *et al.,* 2015). As a result, it seems feasible to develop cultivars with 20–25 percent higher protein content than the present cultivars. However, there have been limited breeding efforts on further improving chickpea protein content. Protein deficit can be alleviated through the production of high-protein cultivars. The linkage has been reported between the flower colour, protein content and seed size. But the linkage was not tight (Kumar *et al.,* 1982). Information on inheritance pattern and relationships of protein content with other traits would help in identifying suitable breeding strategies for developing chickpea cultivars with enhanced protein content, high yield, market preferred grain traits (size, shape and color), and other desired agronomic traits. Information on protein content inheritance patterns and connections with other traits could aid in the development of chickpea cultivars with increased protein content, high yield, market-preferred grain qualities (size, shape, and colour), and other desired agronomic traits. Researchers have increasingly used association mapping to examine complex genetic features in a variety of plant species over the last decade. It is generally observed that popular chickpeas varieties cultivated by the farmers have moderate protein content. The major thought is that the varieties with high protein content have small seed size. Tremendous progress have been made in chickpeas genomics identification of MTAs/QTLs (Varshney *et al.* 2013a and Thudi *et al.* 2014a). Thus, signifies a lot of scope for protein content in chickpeas by MTAs using molecular markers. Therefore, this study has undertaken for identification of SSR markers associated with protein content in a set of chickpeas germplasm.

MATERIALS AND METHODS

The research work was conducted at Research cum Instructional farm, Department of GPB, IGKV, Raipur, Chhattisgarh, during the *Rabi* 2021-22. The material comprised of 37 germplasm and checks T39-1 (Pink), T39-1 (Blue), JG-24, JG-315 and CG Chana 2 (Table 1). The chickpea seeds were sown in the field, in RBD with 2 replications on 7th December, 2021 of 4 rows of 4m length in each

replication with 30 cm x10 cm spacing. Recommended package of practices for chickpea were followed. Random five plants were selected from each of the plot and replications for collecting data of thirteen yield and yield attributing traits at optimal plant growth period on DTF = Days to 50% flowering; DM = Days to maturity; PH = Plant height (cm); HOFP = Height of first pod (cm); PB = Primary branches; SB = Secondary branches; SPP = Seeds per pod; HSW = Hundred seed weight (g); BY = Biological yield (g); Protein = protein content (%); SYP = Seed yield per plant (g). Protein content of seed from each plant was estimated by using standardized procedure by Kjeldhal method using Kelplus-Distyl EM VA. Protein content was obtained by multiplying the total nitrogen content in the seeds by the multiple factor 6.25 (Jones, 1941). Chickpea genomic DNA was extracted from young leaflet of each of the landraces using CTAB method (Dellaporta *et al*., 1983). DNA quantification was quantified on Nano Drop Spectroscopy (*NANODROP, 2000c)*. PCR amplification was done; separation and visualization of amplified products were seen on 5% PAGE; EtBr staining was done and visualized in BIORAD Gel Doc XR+. The size of amplified fragments was determined by 50bp DNA ladder.

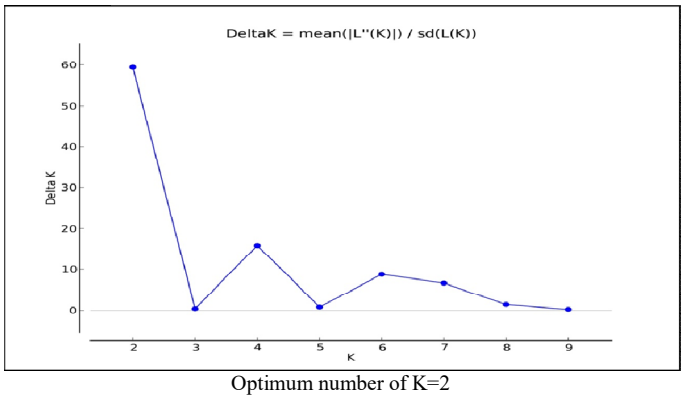
set from one to ten, with 5 independent runs each. Evanno's method (Evanno *et al.,* 2005) was used to estimate the most probable structure number of K. The chickpea lines were classified into various sub-groups with a membership probability threshold (Q) of 0.80 (Zhang *et al.,* 2010b). The admixed group consisted of those lines with Q less than 0.80. The method of association analysis was tackle using the mixed linear model (MLM) method in TASSEL v4.0 (Bradbury *et al.,* 2007). The model MLM_Q+K, using kinship matrix& Q-matrix as the associated variable, was used to identify the marker-trait association. False discovery rate was applied to spot statistically significant loci. Marker trait association at P<0.01 were considered as significant.

RESULTS AND DISCUSSION

During the study, a wide range of crude protein content was observed in chickpea genotypes, ranging from 13.87% (T39-1Pink) to 26.85% (GP15) with an average protein content of 22.41%.

Table 1. Chickpea genotypes with origin

GP No.	Accession No.	Source	GP No.	Accession No.	Source	GP No.	Accession No.	Source
GP2	NC33367	unknown	GP52	IC468839	Raj	GP152	IC272459	Bihar
GP5	IC272496	AP	GP54	IC551991	unknown	GP154	IC328117	MP
GP6	IC272212	MP	GP58	IC468756	MP	GP156	IC552190	unknown
GP8	IC027282	unknown	GP60	ICC3498	unknown	GP159	IC275626	MP
GP10	IC275853	Raj	GP79	IC468727	MP	GP160	IC468600	UP
GP11	IC468840	Raj	GP80	IC486991	MH	GP161	IC327655	UP
GP12	IC468742	MP	GP81	IC770	unknown	GP164	IC327527	MP
GP13	IC327362	TN	GP82	IC512075	Delhi	GP165	IC84017	Delhi
GP15	IC267309	unknown	GP87	IC116340	unknown	GP181	IC305441	Punjab
GP16	IC272401	Bihar	GP91	IC506784	unknown	T39-1 ((P)	Check	Landrace
GP17	IC208294	MP	GP94	EC441751	unknown	T39-1 (B)	Check	Landrace
GP19	IC272196	MP	GP96	IC487505	MP	JG24	Check	MP
GP29	IC348552	MP	GP97	ICC5980	unknown	JG315	Check	MP
GP51	IC268874	UP	GP127	ICC4425	unknown	CG Chana 2	Check	CG



Optimum number of K=2

Figure 1. Estimation of K and population structure. Changes in ΔK value with the number of subpopulations

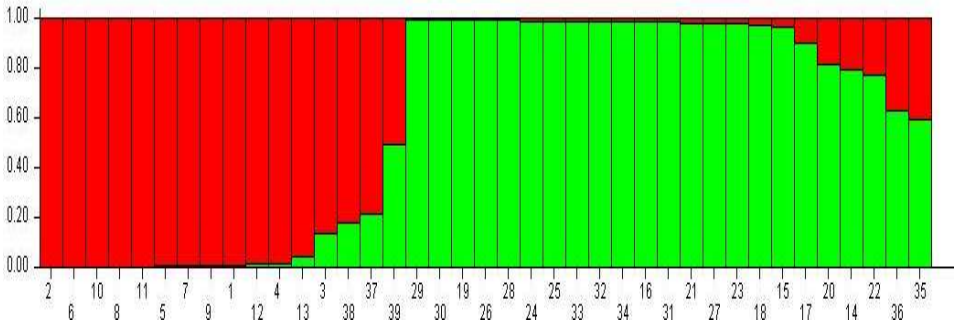


Figure 2. Bar plot showing the population structure of 39 chickpea germplasm accessions based on SSR markers at K=2

A set of 8 SSR markers (C# 2, 3, 5 and 7) were chosen for population structure analysis. The model based program structure v2.3.4 (Earl and von Holdt, 2012) was used to infer the population structure of chickpea accessions using a burn in time of 100,000 and Monte Carlo Markov Chain replicates of 100,000. The number of groups (K) was

Thus, three classes were found high (25-30%), medium (20-25%) and low 10-20%). The four checks namely T39-1(Pink), CG Chana 2, JG 24 and JG 315 recorded low protein content ranging from 13.87 to 16.92%. Moreover, two chickpea genotypes GP-127 (ICC4425) and GP 165 (IC84017) showed less protein content. Thirty two genotypes

comprised the medium category of protein content. IC275853 (25.33%), EC 441751 (25.45%), IC 272496 (25.84%) and IC267309 (26.85%) had high protein (Table 3).

Population Structure analysis: The Bayesian model-based STRUCTURE v2.3.4 program was used to infer population structure of chickpea genotypes. The 39 lines were divided into two sub groups (Fig. 1 and 2) based on the result of Structure Harvester, as delta K kinship was highest at K=2. With population inferred ancestry (Q) = 0.80, 11genotypes were assigned to subgroup POP1, 15 genotypes were assigned to subgroup POP2 and thirteen (13) genotypes namely, GP13, GP 3, GP 38, GP 37, GP 39, GP 18, GP 15, GP 17, GP 20, GP 14, GP 22, GP 36 and GP 35 were assigned to admixture (AD) which has less than <0.80 inferred ancestry (Fig 2). Courtois *et al*. (2012) has successfully detected two subgroups in their study population and assigned varieties into two groups with few admixture lines. Our results are also in conformity with the findings of Borba (2010) suggesting that using structure analysis, the accessions were sub divided into two panels. Likewise, the association of yield traits with SSR markers was undertaken with MLM model, with markers and sub population as fixed factors, and kinship matrix as random factor.

Table 2. Significant marker-trait associations based on MLM model (P>0.05)

Traits	Locus	LG	p_Marker	Rsq_Marker
DM	H2JO9	5	0.0125	0.4417
PH	H2BO61	2	0.0213	0.4413
PH	TR26	3	0.0119	0.4352
HOFP	H2BO61	2	0.0089	0.5202
HOFP	TR26	3	0.008	0.4456
PB	H2JO9	5	0.0155	0.281
PB	SSR1	-	0.0213	0.3916
SB	SSR1	-	0.0481	0.2808
SPP	H2E13	7	0.0301	0.4372
SPP	TR56	3	0.0239	0.2848
HSW	CaM1068	5	5.32E-05	0.839
BY	H2JO9	5	0.0589	0.3901
PROTEIN	CaM1068	5	0.0316	0.5435
SYP	CaM1068	5	0.0487	0.523
SYP	H2BO61	2	0.0215	0.4054
SYP	SSR1	-	0.0134	0.5955

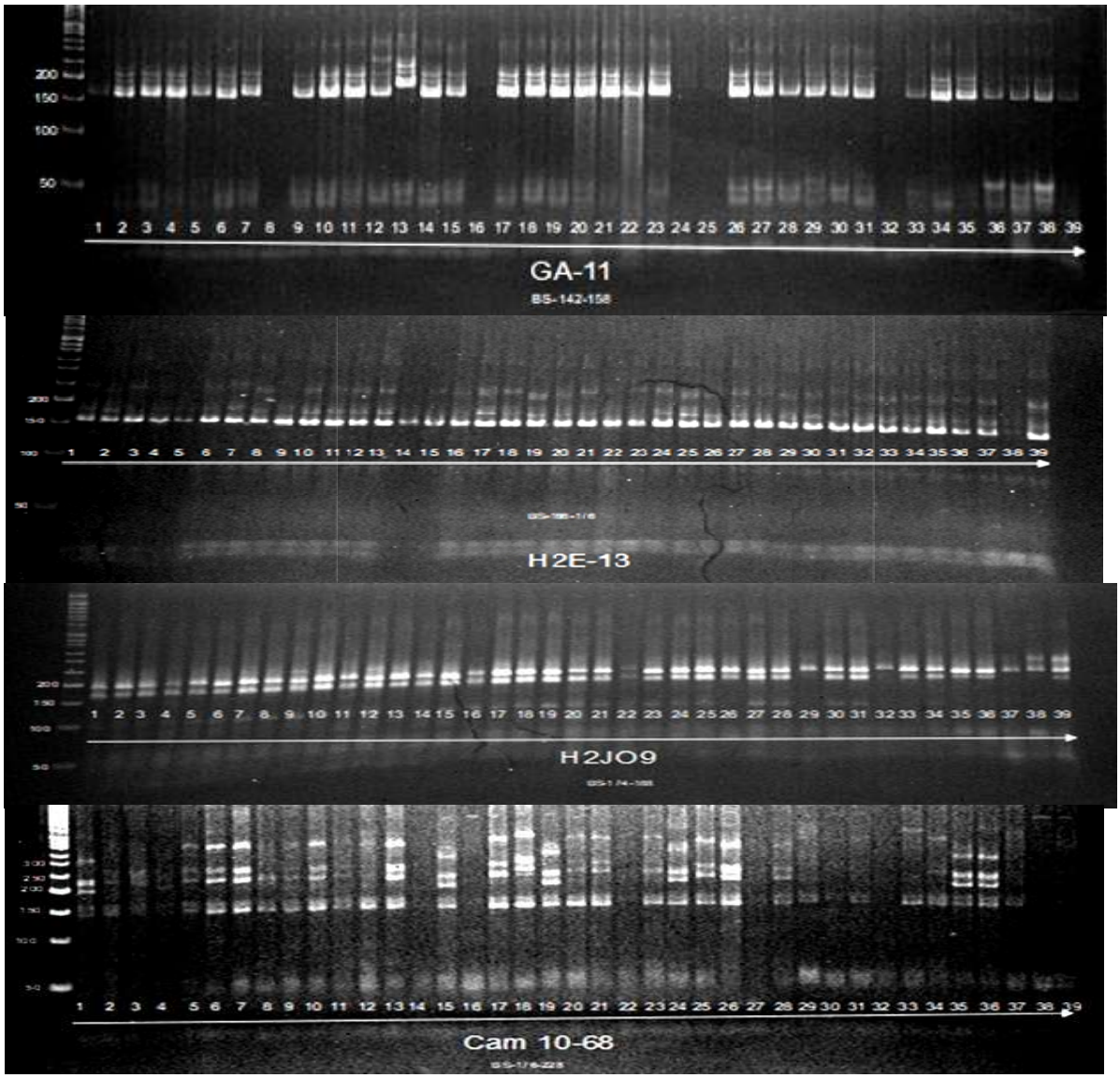


Figure 3. PCR amplification of 39 chickpea germplasm accessions with chickpea SSR makers GA-11; H2E-13; H2JO9 and CaM1068

Marker-trait association: Association analysis between SSR markers and thirteen yield traits and protein content was carried out using Mixed Linear Model (MLM) (Table 2 and fig 3). Seven chickpea SSR markers were found to be tightly linked with the DM, PB, BY, PH, HOFP, SYP, SB, SPP, protein and HSW. These markers covered the linkage groups # 2, 3, 5 and 7. H2JO9 (LG#5) showed tight linkage with DM; PB and BY. H2BO61 (LG#2) showed association with PH, HOFP and SYP. PH, HOFP and SPP showed association with TR 26 (LG#3).

content is significantly positively correlated with DTF (R^2 = 0.32 at $p<0.01$); PH (R^2= 0.28 at $p< 0.01$); PPP (R^2 = 0.26 at $p<0.05$). However, a non-significant and positive association was observed between protein and HSW. HSW is the major component of yield in chickpea. Yield of the crop is always considered to be negatively correlated with its protein content. In our study we observed non-significant association between SYP and protein. Presence of significant QTL's for yield and other related traits has been reported in earlier studies in chickpea.

Table 3. Relationship of chickpea genotypes with flower colour, seed coat colour, seed size, seed yield and protein content (seed and leaf)

GP NO.	Acc. No.	Flower colour	Seed coat colour	HSW (g)	SYP (g)	PLYG (g)	Protein (seed)	Protein (leaf)
GP2	NC33367	Pink	Brown	12.98	2.90	565.00	25.84	35.0
GP5	IC272496	Pink	Brown	12.84	2.14	634.00	24.11	31.1
GP6	IC272212	Pink	Brown	10.23	2.15	437.50	22.24	37.0
GP8	IC027282	Pink	Brown	15.66	4.00	546.00	24.81	30.2
GP10	IC275853	Pink	Dark Brown	12.91	3.45	571.50	25.33	32.2
GP11	IC468840	Pink	Brown	11.18	2.40	617.00	23.65	29.5
GP12	IC468742	Pink	Yellow	16.08	4.15	584.00	21.80	28.7
GP13	IC327362	Pink	Yellow	14.46	3.35	588.50	23.55	27.7
GP15	IC267309	Pink	Brown	18.13	2.30	453.00	26.85	26.6
GP16	IC272401	Pink	Brown	12.18	2.50	594.50	22.03	28.5
GP17	IC208294	Pink	Green	12.51	2.65	450.50	21.95	23.5
GP19	IC272196	Pink	Dark Brown	20.23	2.60	183.50	22.84	28.6
GP29	IC348552	Pink	Brown	13.23	2.65	436.00	24.47	24.6
GP51	IC268874	Pink	Brown	11.98	2.25	553.00	23.21	25.0
GP52	IC468839	Pink	Brown	13.08	2.45	512.50	24.43	28.0
GP54	IC551991	Pink	Brown	35.78	6.85	652.50	24.60	26.0
GP58	IC468756	Pink	Dark Brown	13.73	2.55	267.00	23.11	26.8
GP60	ICC3498	Pink	Brown	17.01	4.05	458.00	20.83	24.2
GP79	IC468727	Pink	Brown	12.54	2.70	455.00	22.70	25.5
GP80	IC486991	Pink	Yellow	13.94	1.55	415.50	23.79	22.8
GP81	IC770	Pink	Yellow	13.41	2.00	589.00	21.91	21.7
GP82	IC512075	Pink	Brown	12.49	1.60	388.50	21.51	25.0
GP87	IC116340	Pink	Brown	13.46	1.75	345.50	22.24	23.3
GP91	IC506784	Pink	Brown	10.46	1.45	149.00	23.88	26.0
GP94	EC441751	Pink	Dark Brown	25.14	4.00	163.00	25.45	27.8
GP96	IC487505	Pink	Brown	12.74	1.50	360.00	24.13	24.3
GP97	ICC5980	Blue	Yellow	10.09	1.85	372.50	23.18	47.8
GP127	ICC4425	Pink	Green	11.44	1.65	453.00	19.20	27.7
GP152	IC272459	Pink	Brown	11.16	2.00	258.00	23.45	23.5
GP154	IC328117	Pink	Brown	12.88	1.30	425.00	23.38	48.5
GP156	IC552190	Pink	Yellow	13.58	3.15	364.50	21.21	48.0
GP159	IC275626	Pink	Yellow	11.78	3.35	449.50	24.52	43.2
GP160	IC468600	Pink	Brown	13.41	2.05	160.00	21.76	44.6
GP161	IC327655	Pink	Brown	13.63	2.45	328.50	22.15	38.3
GP164	IC327527	Pink	Brown	15.13	2.15	438.00	23.08	44.0
GP165	IC84017	Pink	Brown	13.46	2.40	224.00	19.77	48.1
GP181	IC305441	Pink	Dark Brown	11.01	1.90	139.50	22.25	47.8
T39-1 (P)	Check	Pink	Brown	12.00	2.25	203.50	13.87	-
T39-1 (B)	Check	Blue	Brown	8.88	1.95	190.50	24.46	-

SSR1 marker showed linkage with PB, SB and SYP. H2E13 (LG#7) and TR 56 (LG#3) had association with SPP. CaM1068 (LG#5) was the chickpea marker tightly associated with protein, HSW and SYP. Jadhav *et al*., 2015 have also reported the presence of QTL on LG 5 for protein content. When the entire population was tested for association analysis, only one MTA representing one QTL associated with protein marker CaM1068.195 for protein content were detected. In the present study, a total of 16 MTA's from different LG were identified for yield traits. The amount of variation explained by these MTA's ranged from 2.81 to 8.39% in the entire population. Association mapping is therefore sensitive in a way that change in genotype arising due to change in sample sizes may influence the power of detection of a QTL in the entire population. This is expected as the P value tends to be low in the smaller set of genotypes. This is also evident from the smaller values of phenotypic variation explained by the MTA's explained in the entire population as compared to the higher values explained by the MTA's identified in the different populations. Generally, MLM is considered to be more robust as compared to GLM as chances of false positive associations tend to be more with GLM. In our study, it was observed that protein CaM1068.195 (LG 5) has been found to be tightly associated with protein, HSW and SYP. Gowda *et al*., 2011 reported QTL's for yield in chickpea. Significant positive association of protein content has been found with PH. MTA for PH on LG 2 (H2BO61.162) and LG 3 (TR26.205) was recorded. Gowda *et al*., 2011 identified a QTL on LG5 for PH in chickpea. Abbo *et al*., 2005 and Winter *et al*., 2000 reported significant QTLs for protein, PH and HSW with these markers. These examples highlight the importance of LG 2, LG 3 and LG5. Ideally, any genome wide association study should comprise of large number of markers and the genotypes. The summarized data (table 3) depicts about the relationship between the flower colour, seed coat colour, HSW, SYP and protein content both from seeds and as well as from leaves of chickpea genotypes. In our study, the high protein content in seeds were recorded by GP 15 (IC267309) having pink flower, brown seed coat and the translocation of protein content from leaves to seeds was found to be at par followed by GP2 (NC 33367) which had pink flower along with brown seed coat and possessed very small seed size (12.98g). GP2, GP5, GP8, GP 10, GP 15, GP 29, GP52, GP 96, GP 159 and T39-1(P) had pink flower colour and very small seed size ranging from 12.00g to 18.13g. The

seed coat colour also ranged from brown to dark brown colour except GP 159 showed yellow seed coat colour. On contrary to these results, T39-1 (B) exhibited blue colour flower with 8.88g seed size with the seed protein content of 24.46g. There are reports on inheritance of seed size in chickpea, and the main reason of difference is about the parents used in the crossing program. Argikar, 1956 reported that seed size is controlled by a single gene; Ghatge, 1993, Upadhyaya *et al*., 2006, Hosain *et al*., 2010b and Malhotra *et al*., 1997 suggested that the trait is governed by polygenes. The flower colour, seed coat colour showed varying colour intensities. This suggests the pleiotropic effects of gene (s) on these traits. T 39-1 with blue flower had on an average small seed and higher protein content than the pink flowered (T39-1) with (HSW=12g); 13.87% protein content. The high protein content in blue flower could be because of their reduced seed size compared to pink flower. Kumar *et al.,* 1982 also found that blue flowered plants with small seeds had higher protein content compared to pink flowered with larger seeds. They suggested linkages between genes for flower colour, protein content and seed weight. It was also reported by Atta *et al*., 2008. Saxena *et al*., 1987 suggested that breeding lines combining high protein content with medium size can be successfully developed. A significant association of protein content with flower colour, seed coat colour and seed shape suggests the development of chickpea cultivars with high protein content and desired seed traits (size, shape and colour) would require large segregating populations and the selection of desired recombinants. Moreover, the identification and evaluation of trait linked genes through molecular markers can also provide a strongest tool to breeders for chickpea yield improvement. Importantly, seed weight was also proposed as an accurate measure of chickpea seed size (Upadhyay *et al*., 2006). A large seed size variation exists within and between chickpea types, with some desi types as large as Kabuli types and some Kabuli types as small as desi types (Hossain *et al*., 2010). It is therefore, suggested that molecular markers could be useful for the characterization and grouping of germplasm on the basis of their origin and performance (Nissar *et al*., 2008, 2009). GP 10, GP 94, GP 2 and GP 15 had high protein content. Seven chickpea SSR markers were found to be tightly linked with the DM, PB, BY, PH, HOFP, SYP, SB, SPP, protein and HSW. These markers covered the linkage groups # 2, 3, 5 and 7. CaM1068 (LG#5) showed tightly linked to seed protein, HSW and SYP.

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