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RESEARCH ARTICLE

Marker trait association for biological nitrogen fixation traits in an interspecific cross of chickpea (*Cicer arietinum* × *Cicer reticulatum*)

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Abstract
A set of 165 Recombinant inbred lines (RILs) derived from an interspecific cross of chickpea was used to identify QTLs for key biological nitrogen fixation (BNF) traits. The phenotyping of BNF and related traits was done at two different agroclimatic zones viz., Central plain zone (Ludhiana) and Sub-Mountainous undulating zone (Gurdaspur) for 2 consecutive *rabi* seasons (2018–2020). Wild parent *C. reticulatum* ILWC292 showed significantly high performance in terms of biological nitrogen fixation (BNF) traits over the cultivated *C. arietinum* GPF-2. The triple interaction of genotypes × locations × years was significant (p 0.05) for all BNF traits in parental lines. Highly significant positive correlation was obtained between grain yield and key growth and symbiotic parameters at both the sites. Phenotypic analysis revealed nodule dry weight and leghaemoglobin content as key traits for BNF efficiency and contrasting DNA bulks were constituted on the basis of these traits. Out of 535 SSR markers, 139 exhibited polymorphism between the parental lines on polyacrylamide gel electrophoresis. A total of 30 SSR markers showed polymorphism between the higher and lower bulks for nodule dry weight and leghaemoglobin content. Out of these, 20 SSRs did not show any segregation distortion in RIL population as determined by chi square analysis ($p < 0.05$) and were used for quantitative trait loci (QTL) analysis. Using QTL cartographer, markers- CAGM02697, CAGM09835, CAGM09777, CAGM09227, CAGM09021, CAGM08679 were found linked with QTLs for BNF. These markers can be validated further for identification of genes for BNF traits and marker assisted selection in chickpea. To the best of our knowledge this is the first report on identification of genomic regions associated with key BNF traits in chickpea across different agro-climatic zones.

Keywords Biological nitrogen fixation · *Cicer reticulatum* · *Cicer arietinum* · Leghaemoglobin · Nodulation · SSR · Quantitative Trait Loci

Introduction

Chickpea (*Cicer arietinum* L.) is an important cool season (*rabi*) legumes cultivated and consumed across the globe particularly in the Afro-Asian regions. It has a diploid genome (2n = 16) of size 738 Mb. India is the world leader in chickpea production contributing to 61.49% of total global produce, with an average productivity of 0.951 t/ha[2] (Goyal et al 2021). A symbiotic association of chickpea with the group of bacteria (collectively referred to as rhizobia) is the key contributor to atmospheric nitrogen fixation in the *rabi* season, the process known as biological nitrogen fixation (BNF). BNF is crucial for sustainability of the agricultural ecosystems, especially under the current climate change scenario, as they lower the requirement and consumption of nitrogen (N) fertilizers thereby controlling greenhouse gas emissions and water pollution.

Plant breeding relies on exploitation of natural variation in a species. Conventional plant breeding and crop improvement schemes evaluate and exploit the inherent genetic variability present in the germplasm. Phenotypic traits associated with BNF are of quantitative nature and therefore are tiresome to improve via conventional breeding approaches

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(Dwivedi et al. 2015). Crop improvement programs have not yet targeted BNF traits due to laborious phenotyping, complex genetic control and amalgamated role of varied growth and nodulation parameters in an efficient BNF process. However, genome of chickpea has been sequenced and abundant SSRs were generated, which have greatly facilitated the mapping of genomic regions associated with quantitative traits like BNF.

With the use of modern molecular tools and pre-breeding activities, some useful traits governing BNF can be introgressed into cultivars, which can be further used to augment the yield of chickpea. However, the morphological parameters for precise phenotyping of chickpea germplasm for high BNF efficiency have not been standardized. The quantity of N assimilated by the legumes and rhizobia during the BNF process is found to be closely related to the leghaemoglobin content of nodules (Wilson and Reisenauer 1963; Lakshmanarao and Singh 1983). Genotypic variation for root traits, both morpho-physiological (root size) as well as functional (exudates), and nodulation capacity have been observed in chickpea genotypes (Ali et al. 2002; Mathew and Shimelis 2022; Shi et al. 2022). Genetic analysis indicated the combination of additive and non-additive genetic components for inheritance of all the nodulation and yield characters in chickpea (Verma and Waldia 2013).

BNF is a complex trait and thus is of quantitative inheritance. None of the morphological parameters identified for BNF have shown simple or qualitative inheritance (Biljon and Sifi 2021). Improvement of N_2 assimilation can be achieved by optimizing the cultivation conditions and alleviating any stresses or by breeding and cultivation of genotypes with improved assimilation efficiencies (Yang et al. 2019). The expression of quantitative traits like BNF is critically influenced by environmental conditions and considered as one of the major hurdles in their improvement through breeding.

Low level of variation in cultivated *Cicer arietinum* necessitates the exploration of secondary and tertiary gene pool for genetic improvement. The earlier studies on crossability among wild annual *Cicer* species for broadening the genetic base advocate that *C. reticulatum* Ladiz. and *C. echinospermum* P.H. Davis species can be inter-crossed with the cultivated *C. arietinum* species (Saxena et al. 2014). Therefore, evaluation of wild *Cicer* species will greatly aid in trait discovery related to BNF capacity in chickpea. Pre-breeding and marker assisted introgression of high BNF efficiency into cultivated chickpea could augment the yield and protein content.

Finite insight is available on the legacy of BNF in an important legume crop like chickpea. This complex phenomenon of N_2-fixation is an integrated effort of both chickpea host and rhizobia via multiple genes of both the partners. Extensive research has been undertaken to explore the genetic basis of BNF in regards to rhizobial microsymbiont including the bacterial genes involved in the process viz., *nif* (Dasgupta et al. 2021; Li and Chen 2020), *nod* (Geddes et al. 2020; Del Cerro et al. 2019), *fix* (Ledermann et al. 2021; Burghardt 2020) and *nol* (Chen et al. 2021; Thomas and Rahman 2020) but the chickpea host genes are still unexplored. Genetic control of BNF, nitrogenase activity and nodulation traits are directed by additive or non-additive genes, with some proof of epistatic interactions (Farid 2015; Jingade and Ravikumar 2018).

Molecular mapping of genomic regions or QTLs (Quantitative Trait Loci) governing a quantitative trait is advantageous over the conventional quantitative genetic analysis. Nevertheless, few QTL and candidate genes underlying these QTLs governing nodulation traits have been distinguished in model legumes, soybean and rice (Donde et al. 2020; Yang et al. 2019; Hwang et al. 2014, Santos et al. 2013). Further, molecular tools may also be helpful to cater the major limitation of poor knowledge about genetics of quantitative traits viz. protein content in grains and BNF in chickpea (Torres et al. 2015). Most QTLs concerning BNF overlap with QTLs for protein content (Santos et al. 2013), but to date, no studies have addressed simultaneous increase in both traits. Till date, little information is available on genetic variation in wild and cultivated chickpea with respect to BNF and protein traits. Hence, to understand the genetic basis of these two traits in combination determining the efficiency of complex BNF process is necessary. A set of wild *Cicer* have already been explored for BNF traits in Pulses Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The interspecific mapping population derived from high (wild *Cicer*) and low nodulating (cultivated *Cicer*) lines were available with the department. In present study, the Recombinant Inbred Lines (RILs) derived from the cross of wild and cultivated *Cicer* were evaluated for key BNF traits and genotyping was done for identification of regions associated with efficient N_2 fixation in chickpea.

Material and methods

Materials used under field trials

The choice of the experimental sites was made covering two different agroclimatic zones viz., Central Plain zone (Pulses Research farm, Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana, 30°54′3.4740″N and 75°51′26.1972″E) and sub mountain undulating zone (Regional Research station, PAU, Gurdaspur, 32°2′30.9948″N and 75°24′19.2024″E) over two winter seasons (2018–2020). The two parental lines used were cultivated *Cicer arietinum* (GPF-2) and wild *Cicer*

reticulatum (ILWC292) along with 165 $F_{7:9}$ Recombinant Inbred Lines (RILs) of chickpea. Each line was sown in two rows with net plot size of 1.8 m^2. Seeds of chickpea RILs and parental lines were obtained from the Pulses Section, Department of Plant Breeding and Genetics, PAU, Ludhiana. Sixty seeds per row were used in experiment conducted at two locations. Pre-sowing irrigation was applied to keep the soil at an adequate moisture level in the experimental field. A disc harrow was used twice to prepare the field for sowing, followed by a farm cultivator, and a planker. Weeds were controlled using pre-emergence application of pendimethalin @ 0.75 kg ha^{-1} using 500 L of water and hand weeding as per the crop need. Sampling of soil samples was done as homogenous units with an auger to a plough depth of 15 cm from several locations of field in a zig-zag manner. The soil samples from both the locations were analyzed for physicochemical and nutrient characteristics both before sowing and after harvesting over years to screen for pH, electrical conductivity (EC), organic matter (OM), total nitrogen (N) (Subbiah and Asjia 1956), phosphorus (P) (Jackson 1973) and potassium (K) content (Jackson 1967). Temperature variation of two locations over the entire study period was also recorded.

Phenotyping of parents and RILs for BNF traits

Five representative plant samples of parental lines and RILs were sampled at vegetative and flowering stages at each location for investigating growth (plant height, root length, chlorophyll content and plant biomass), BNF (nodulation, nodule dry weight and leghaemoglobin content) and yield related traits. Intact plants were uprooted from soil without damaging the roots and parameters viz., plant height (cm), root length (cm), nodule number per plant and plant biomass were recorded. Plants were removed gently from the soil and placed on a sieve. The roots were carefully washed in flowing water, nodules removed with forceps from the roots, counted and expressed as number of nodules $plant^{-1}$ by taking their average. Nodules were further subjected to air-drying in an oven at 60 °C till the constant weight was obtained for recording their dry weight and expressed as weight of nodules per plant.

Chlorophyll content of leaf was estimated using protocol described by Witham et al. (1971). One gram of healthy leaves was extracted with 80% acetone and centrifuged for 10 min at 5000 rpm. The Absorbance of the chlorophyll extract was recorded on Elico Vis spectrophotometer using a solvent blank at 645 nm and 663 nm and the obtained values represented as mg/g fresh weight of leaves. Leghaemoglobin content of nodules was determined using Drabkin's solution as suggested by Wilson and Reisenauer (1963). One gram of fresh nodules was extracted with 3 ml Drabkin's solution thrice and centrifuged at 10,000 rpm for 15 min. The absorbance of clear solution was read at 540 nm using Elico Vis spectrophotometer and leghaemoglobin content calculated as mg/g fresh weight of nodules. Total seed protein content was assayed using method described by Lowry et al. (1951). One ml of supernatant obtained after crushing the seeds in phosphate buffer (pH 7.0) was mixed with 5 ml of reagent C (50 ml of reagent A {2% Na_2CO_3 in 0.1 N NaOH} + 1 ml of reagent B {0.5% $CuSO_4$ in 1% Sodium citrate} and 0.5 ml of reagent D (1:1 phenol reagent and distilled water). Deep blue color of supernatant was measured at 520 nm and total seed protein content estimated.

Parental polymorphism

The genomic DNA of wild *Cicer reticulatum* ILWC292 and cultivated *Cicer arietinum* GPF-2(parents) and RILs was extracted using CTAB method (CetylTrimethyl Ammonium Bromide) (Doyle and Doyle 1987). A set of 535 co-dominant microsatellite (SSR) markers from chickpea genome, available in public domain (Huttel et al. 1999; Winter et al. 2000; Cobos et al. 2005; Sethy et al. 2006; Varshney et al. 2013; Jadhav et al. 2015) were used to detect the polymorphism between two parents. The primer pairs were synthesized from Integrated DNA Technologies (IDT). The Polymerase Chain Reaction (PCR) amplification was performed to check the variation in the wild and cultivated parental lines using SSR markers. After PCR amplification, 2% agarose gel was used to visualize the amplified product and gel was visualized in gel documentation system (Alpha Imager, USA). The SSR markers were subjected to polyacrylamide gel electrophoresis (PAGE) (6% polyacrylamide gel) to detect the slightest polymorphism between the parental lines at an increased resolution.

Bulked segregant analysis for key BNF traits

High and low DNA bulks were made for key BNF traits viz., nodule dry weight and leghaemoglobin content on the basis of phenotypic data of RILs over two years at both the experimental sites. The contrasting bulks consisted of pooled DNA of high and low performing RILs for key BNF traits. The markers, showing polymorphism between parents were used for PCR amplification of two DNA bulks. Markers showing polymorphism between the two bulks were further used to genotype the 165 RILs.

Test for segregation distortion of polymorphic SSR markers

The 165 RILs along with their parents were subjected to genotyping with SSR markers showing polymorphism in high and low DNA bulks for key BNF traits. Test of segregation distortion to test the goodness of fit was done

using chi-square and p value < 0.05. A similarity coefficient based on neighborhood joining method was used in SSR analysis and the genotypic data of SSR markers was analyzed with Principal Coordinate Analysis (PCoA).

Marker trait association for key BNF traits

Marker trait association analysis (Jansen and Stam 1994; Zeng 1994) for finding the markers linked to key BNF traits was performed by Win QTL Cartographer 2.5.0. on the basis of genotypic and phenotypic data of 165 RILs. Both main and epistatic effect genomic regions were determined in the study using same software (Wang et al. 2012). Marker trait analysis facilitates automatic co-factor selection by afterward/backward regression. The threshold log likelihood ratio (LOD) score was estimated with 1000 permutations to declare significance at 0.05 for the trait evaluated.

Statistical analysis

Phenotypic data in triplicates was statistically analyzed using an analysis of variance (ANOVA) ($p < 0.05$). Pearson correlation analysis ($p < 0.05$) was carried out to measure the strength of association between two BNF variables and the direction of the relationship. Marker segregation data obtained from scoring of polymorphic SSR markers in the segregating RIL population was used for molecular mapping of varied BNF traits. All data analysis was carried out by using R-studio and SAS softwares. Marker trait association analysis was carried out using WinQTL Cartographer (Wang et al. 2012).

Results

Environmental variation at two locations

The soil at Ludhiana was sandy loam in texture developed under semi-arid and warm to hot climate. At RRS, Gurdaspur, soil was found to be alluvial, clayey, deep and fine grained developed under sub-moist and cool to warm temperate climate. Significant variation existed in soil samples of both the locations for varied physico-chemical and nutrient parameters. On pooled mean basis of two *rabi* seasons at the time of harvest, soil samples from Gurdaspur were rich in total organic matter (1.03%), nitrogen (97 kg/ha), potassium (138 kg/ha) and phosphorus (20.6 kg/ha) in comparison to soils at Ludhiana (0.79%, 90.5 kg/ha, 129 kg/ha and 19.5 kg/ha, respectively).

Phenotypic variation among parents and RILs

The two parental lines *C. reticulatum* (ILWC292) and *C. arietinum* (GPF-2) varied significantly for key BNF traits. ANOVA ($p < 0.05$) revealed significant variation between two *Cicer* parents for growth, symbiotic and yield traits at both locations over the years. Wild parent ILWC292 showed better root length, chlorophyll content, shoot- root dry weight, nodule dry weight, leghaemoglobin content as compared to GPF-2 at both the seasons (Fig. 1). Although cultivated parent GPF-2 showed high number of nodules than the wild parent but nodulation ratio (nodule dry weight/nodule number) was found higher in wild ILWC292 over GPF-2. The total protein content of seeds was superior in wild parent ILWC292 over cultivated *Cicer* at Ludhiana and Gurdaspur, respectively (Fig. 1). In contrast, cultivated *Cicer* parent GPF-2 had high grain yield in comparison to wild ILWC292 at both Ludhiana and Gurdaspur, respectively.

Significant phenotypic variation was observed in 165 RILs for all key BNF traits across the locations over the years. The distribution of values for all growth traits showed a normal distribution. The mean value for plant height among RILs was higher than the mean values for both the parents under different environments (Table 1). The mean root length in *Cicer* RILs was superior at Ludhiana by 1.22-folds over Gurdaspur. The maximum and minimum values for chlorophyll content of leaves in chickpea RILs were in range with the extremes of the two parental lines at both the locations. The range of shoot- root dry weight in the RILs was extended at Ludhiana while it was narrow at Gurdaspur. Range of chickpea RILs for nodulation traits exceeded that of the parental lines. The mean nodulation at Ludhiana was double as that obtained at Gurdaspur (Table 1). High mean value was noticed in nodule dry weight in all the RILs at Ludhiana during first season but in second season mean nodule dry weight was higher at Gurdaspur.

Correlation analysis

At Ludhiana, the phenotypic correlation coefficients among key BNF traits ranged from -0.016 to 0.53. Plant height significantly correlated with grain yield, chlorophyll content, shoot dry weight, and nodule number (Fig. 2A). Grain yield was observed to be positively correlated with nodule number, chlorophyll content and shoot dry weight. Nodule number correlated with nodule dry weight and shoot dry weight at $p < 0.05$. At Gurdaspur, the phenotypic correlation coefficients among key BNF traits ranged from −0.02 to 0.49. Plant height showed significant correlation with grain yield, root dry weight and chlorophyll content. Nodule number was positively correlated with nodule dry weight, leghaemoglobin content, root dry weight and grain yield ($p < 0.05$). Root dry weight and nodule number were also

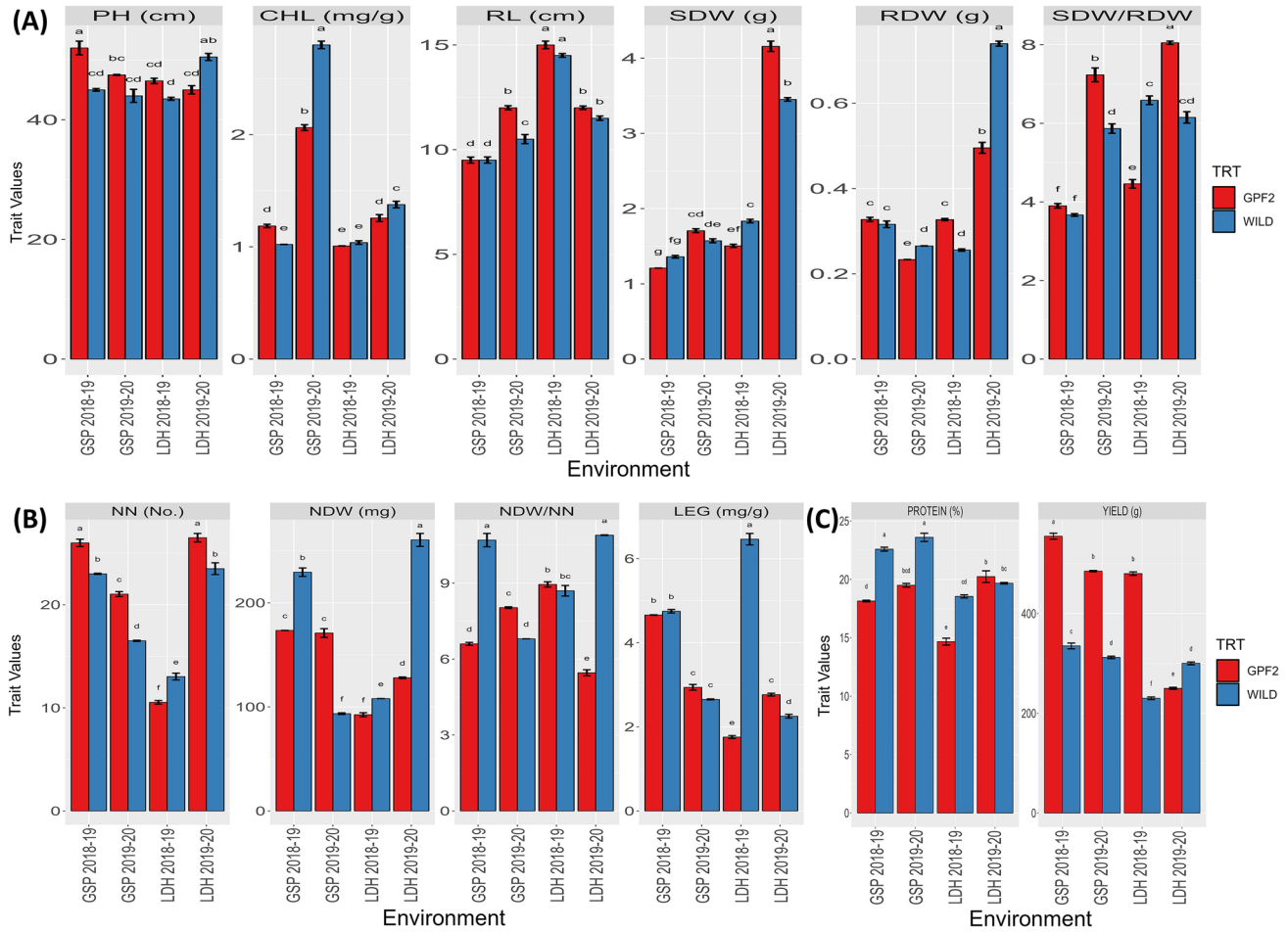

Fig. 1 Multilocation analysis of phenotypic variation in *C. arietinum* GPF-2 and *C. reticulatum* ILWC292) for key BNF traits over the years. Means depicted by the same alphabet are not statistically significant according to Tukey's grouping for least square means ($p < 0.05$). Where, LDH- Ludhiana, GSP-Gurdaspur, PH- plant height, CHL-chlorophyll content, RL- root length, SDW- shoot dry weight, RDW- root dry weight, NN-nodule number, NDW- nodule dry weight, LEG-leghaemoglobin content

Table 1 Effect of locations and seasons on key BNF traits in *Cicer* RILs

Traits	Ludhiana				Gurdaspur			
	2018–2019		2019–2020		2018–2019		2019–2020	
	Range	Mean	Range	Mean	Range	Mean	Range	**Mean**
Plant height (cm/plant)	25.00–52.00	41.08	26.00–159.00	41.58	32.5–54.25	44.01	28–56	42.72
Root length (cm/plant)	10.08–19.92	14.61	8.00–20.00	12.75	7.83–18	11.02	7.5–13	10.37
Chlorophyll content (mg/g fresh weight of leaves)	0.73–1.22	1.02	1.03–2.82	2.02	0.77–1.5	1.07	2.047–3.048	2.53
Shoot dry weight (g/plant)	0.23–4.10	1.25	0.70–7.25	3.21	0.07–2.41	0.50	0.5—2.24	1.39
Root dry weight (g/plant)	0.08–0.64	0.24	0.16–1.50	0.57	0.09–0.4	0.20	0.124–0.502	0.29
Number of nodules per plant	5.83–70.94	28.58	9.00–50.00	28.25	8.06–61.83	20.51	6–26	14.03
Nodule dry weight (mg/plant)	21.50–346.00	138.28	41.00–483.50	182.58	27–348.5	103.62	44–615.5	198.81
Leghaemoglobin content (mg/g fresh weight of nodules)	1.65–13.37	5.27	0.73–3.77	2.47	1.44–7.99	4.42	1.45–5.42	3.26
Total seed protein content (%)	9.55–27.67	17.48	9.50–26.80	18.26	7.77–31.14	19.93	10.1–29.2	20.86
Grain yield (g)	10.00–750.00	374.13	81.00–448.00	265.80	75–635	401.00	120–496	316.70

Values are mean of three replications

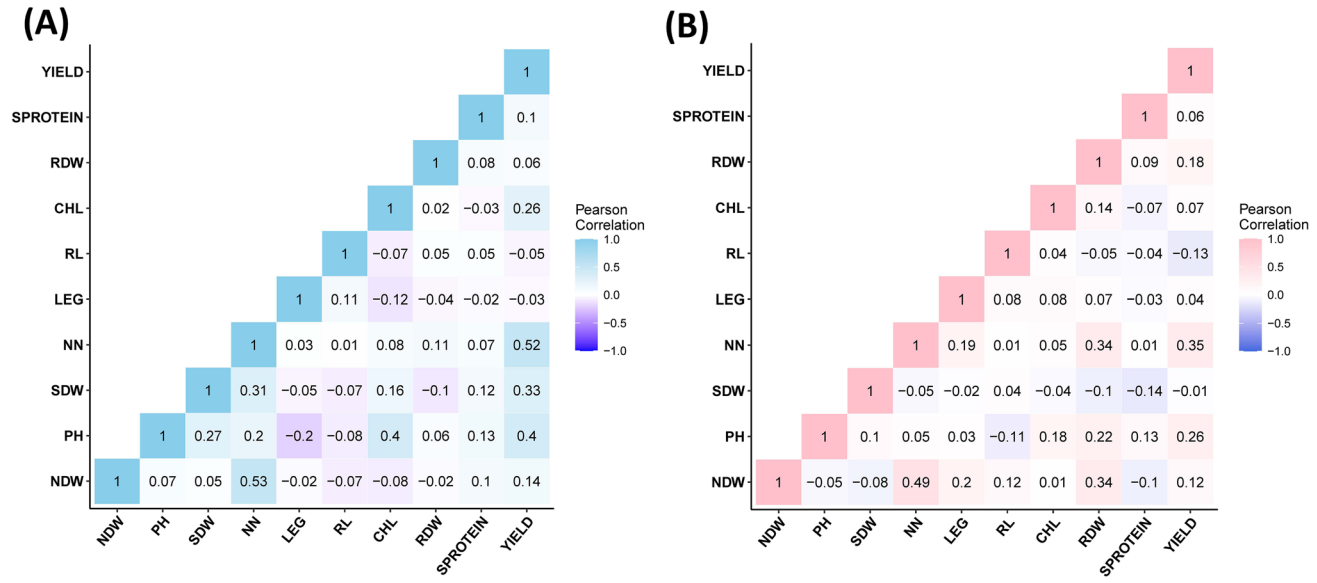

Fig. 2 Pearson correlation analysis for key BNF traits at **A** Ludhiana and **B** Gurdaspur. Where, NDW- nodule dry weight, PH- plant height, SDW- shoot dry weight, NN-nodule number, LEG-leghaemoglobin content, RL- root length, CHL-chlorophyll content, RDW- root dry weight, NDW- nodule dry weight, SPROTEIN- seed protein content

found to be positively correlated (Fig. 2B). Key symbiotic traits viz., nodule dry weight and leghaemoglobin content were significantly correlated with r value 0.20 highlighting their integrated role in the complex BNF process. Both the traits are considered as an index of effective N_2 fixation in chickpea, hence are used in the study to determine regions governing complex BNF process.

Parental polymorphism with SSR markers

The two *Cicer* parental lines *Cicer reticulatum* (ILWC292) and *Cicer arietinum* (GPF-2) were screened for polymorphism using 535 SSR markers (covering all 8 chromosomes of chickpea) using agarose gel electrophoresis. No polymorphism was obtained on agarose gel (up to 4% concentration) between the two parental lines. This might be attributed to the narrow genetic diversity and narrow gene pool of *Cicer*. Hence, polymorphism between the parental lines was further assessed on polyacrylamide gel electrophoresis (PAGE) because it provides higher resolution (< 10 bp difference). The PAGE analysis revealed 139 SSR markers polymorphic between two *Cicer* parental lines (Fig. S2) (Table S1). These polymorphic markers were further used for bulked segregant analysis of key BNF traits.

Bulked segregant analysis for key BNF traits

High and low DNA bulks of RILs were prepared on the basis of phenotypic data obtained for key BNF traits viz., Nodule dry weight and leghaemoglobin content (in experiment 1) and were chosen for the constitution of bulks. Frequency distribution graphs depicted continuous variation in the large RIL population for both nodule dry weight and leghaemoglobin content. In addition, positive correlation was noticed between nodulation traits and grain yield further highlighting their role as key determinants in BNF process. Keeping this in view, contrasting bulks (high bulk and low bulk) were constituted for both traits based on the phenotypic values by selecting RILs with extreme phenotypes and pooling their DNA samples in equal ratio (Table 2). A set of 139 polymorphic SSR markers identified through parental polymorphism were employed on both HB and LB for studying the polymorphism between both constituted bulks. Of the 139 polymorphic SSR markers, 30 markers were found polymorphic between low and high nodulating DNA bulks (Fig. S3). The selected 30 SSR markers were used for genotyping of 165 RILs for further analysis.

Genotyping of RIL population with polymorphic markers

The 30 SSR markers identified for polymorphism between HB and LB were further applied on 165 RIL population to reveal the co-segregation of marker-trait analysis. The genotypic data of chickpea RILs obtained using SSR markers was recorded as 'A' for high nodulating parent (*Cicer reticulatum* ILWC292) and 'B' for low nodulating parent (*Cicer arietinum* GPF-2) (Fig. 3). The frequency of A- and B- DNA amplicons were recorded and segregation analysis was done. Goodness of fit of observed segregation ratio to the expected

Table 2 Mean values of key BNF traits in extreme bulks constituted for bulked segregant analysis

Nodule dry weight (mg/plant)				Leghaemoglobin content (mg/g fresh wt. of nodules)			
Low bulk		High bulk		Low bulk		High bulk	
RIL16	87	RIL12	269	RIL25	1.79	RIL5	5.48
RIL57	45	RIL14	320	RIL57	2.82	RIL96	6.04
RIL128	45	RIL23	251	RIL58	1.59	RIL98	5.19
RIL134	27	RIL32	113	RIL61	3.42	RIL108	6.07
RIL146	21.5	RIL77	332	RIL72	1.72	RIL120	4.96
RIL158	70	RIL105	320	RIL88	1.35	RIL125	4.77
RIL169	50	RIL108	109	RIL91	2.32	RIL128	6.42
RIL178	40	RIL125	496	RIL134	1.86	RIL136	7.22
RIL188	34	RIL133	314	RIL138	3.01	RIL160	4.19
RIL196	49	RIL198	259	RIL156	1.92	RIL167	7.72
Mean	46.8		278.3		2.18		5.81

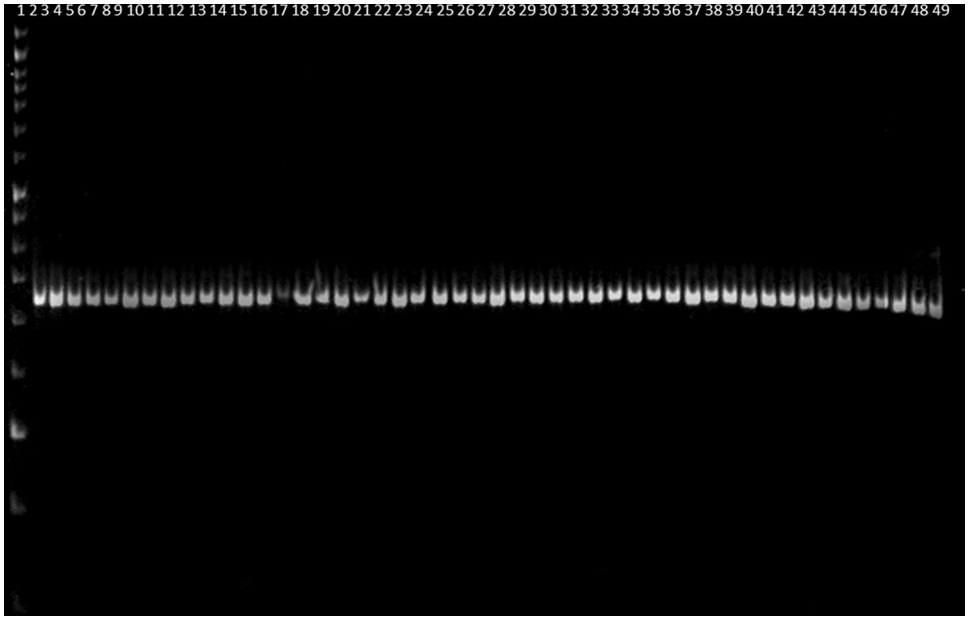

Fig. 3 Segregation of SSR markers in mapping population derived from *C. arietinum* × *C. reticulatum*. Lane 1 depicts 50 bp DNA ladder and lanes 2 to 49 depict segregation obtained in mapping population with polymorphic SSR marker

ratio demonstrated that majority of SSRs did not significantly deviate from expected 1:1 ratio ($p < 0.05$). Out of 30 markers, 20 markers segregated in 1:1 ratio with chi square value ranging from 0 to –3.327 at $p < 0.05$ (Table S2). These twenty markers were further used for marker trait analysis.

Cluster and principal coordinate analysis

The allelic data obtained with 30 SSR markers across 165 RIL populations, was computed to obtain dendrogram which clearly differentiated the lines. Based upon the phylogenetic relationship obtained from SSR marker loci, 165 RILs were grouped into four major clusters (1–4) (Fig. 4). The first cluster consisted of 42 *Cicer* lines which were further divided into two major subclusters. Subcluster 1a included seven RILs while subcluster 1b comprised of 35 RILs. The cultivated *Cicer* parent GPF-2 was grouped in subcluster 1a. The second cluster comprised of 24 RILs. The third cluster was smallest of all including only 4 RILs. The 4th cluster was the largest among all the clusters with 95 RILs. The wild *Cicer* parent ILWC292 was included in cluster 4 (Fig. 4). Distinct clustering pattern revealed diverse nature of both the wild and cultivated parental lines of chickpea.

The Principal Co-ordinate analysis (PCoA) differentiated all 165 chickpea RILs and the two parental lines. The first and second component axis accounted for 50.79 and 15.42 percent of total variance (Fig. 5). The PCoA categorized the RILs into four groups involving different RILs similar to UPGMA-neighbor joining clustering (Fig. 5). Biplot PCA showed correlation with UPGMA based phylogenetic tree with respect to grouping of chickpea RILs.

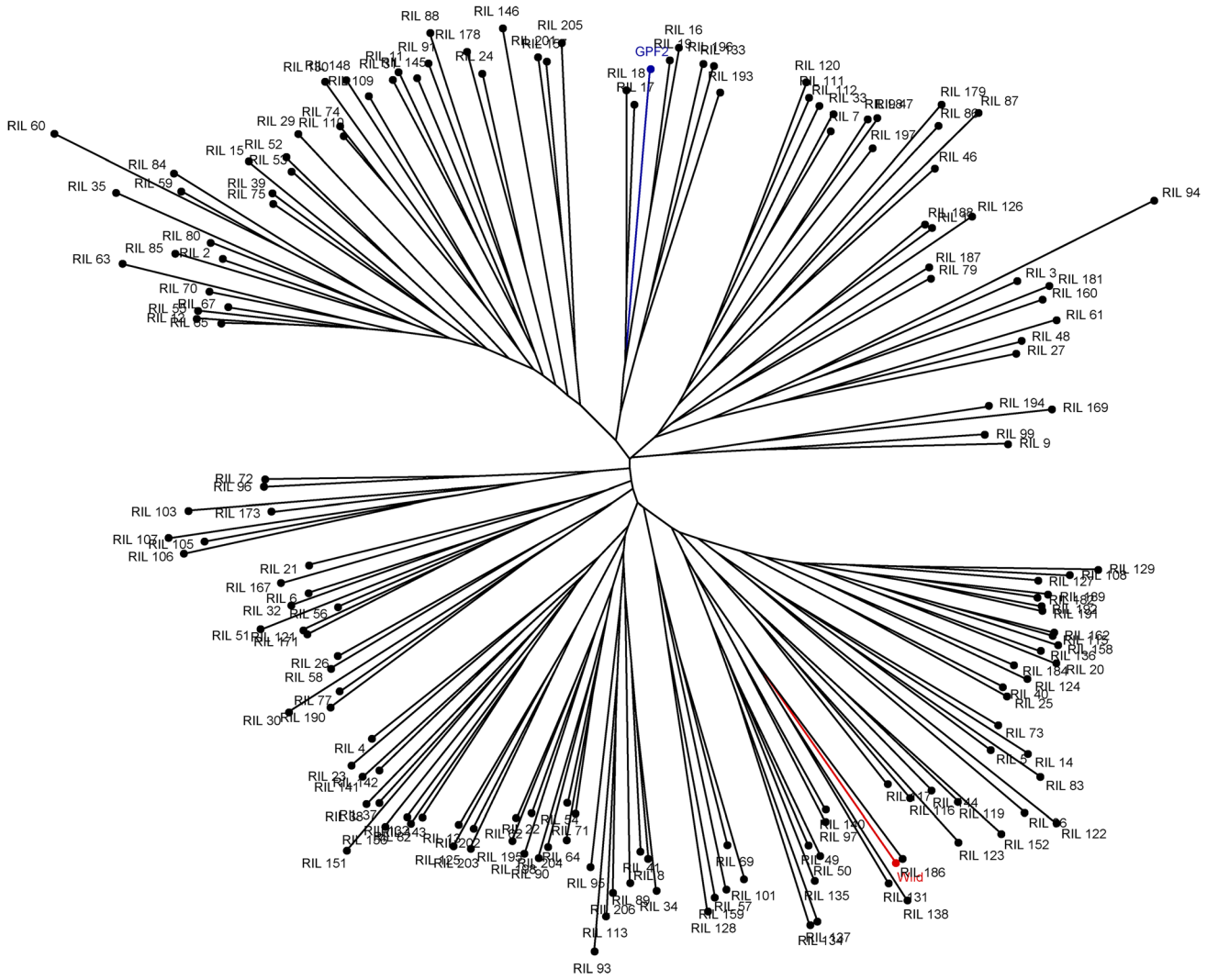

Fig. 4 Dendrogram showing relationship between 165 *Cicer* RILs as revealed by cluster analysis based on UPGMA neighbor-joining

Marker trait association analysis for key BNF traits

Twenty SSRs without any segregation distortion were further used for marker trait analysis using Win QTL cartographer. Different markers were found linked to key traits governing BNF at both locations.

At Gurdaspur, markers covering diverse chromosomes of chickpea were found significantly linked to key growth, symbiotic and yield traits governing BNF. The markers and their sequences along with the percent phenotypic variation explained by each marker are given in Table 3. For instance, marker CaGM08679 (Chromosome {Ca2}) was found linked with both root length and total seed protein content explaining about 3.4% and 2.7% phenotypic variations in the traits, respectively. Marker CaGM09021 (Ca2) was found linked with shoot dry weight (SDW), SDW/RDW, nodule dry weight (NDW), and NDW/NN explaining 0.23%, 2.73%, 1.65% and 0.55% phenotypic variations respectively for the growth and nodulation traits. The minor phenotypic variation explained by the linked markers depicted the quantitative nature of BNF traits and role of multiple factors viz., environment, soil and genotypes on their expression. Two markers viz., CaGM08632 and CaGM09777 (Ca3) were found to be linked with nodule dry weight and ratio of nodule dry weight/nodule number (NDW/NN) explaining 3.5% and 3.1% variations in trait respectively (Table 3). Overall, 9 SSR markers were found associated with various BNF traits in chickpea.

At Ludhiana, 2 markers viz., CaGM02697 (Ca1) and CaGM09835 (Ca3) were found linked with both plant height and grain yield showing 3.4% and 4.0% and 3.1% and 1.5% variation in traits respectively. Pearson correlation analysis revealed highly significant correlation between plant height and grain yield at Ludhiana. The same is further validated

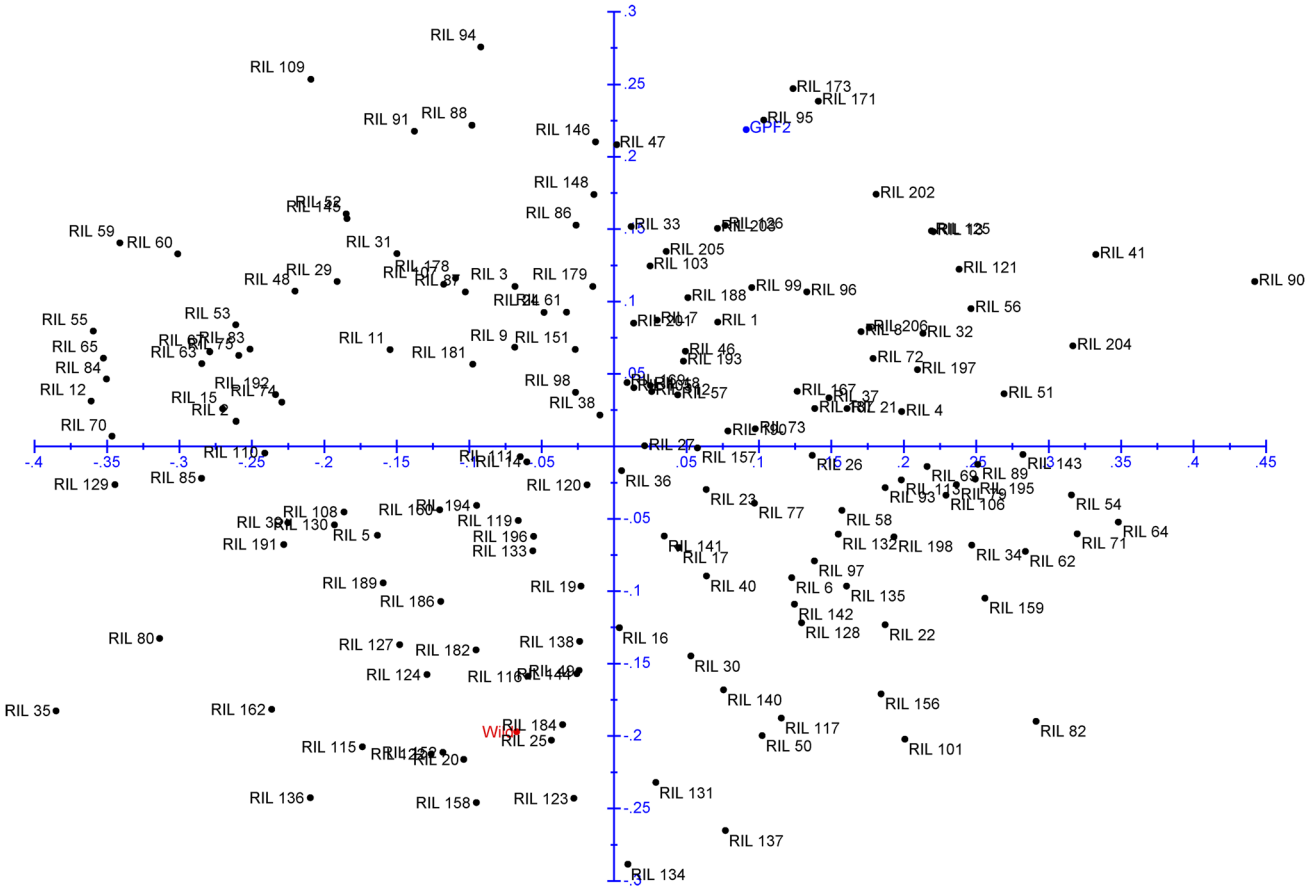

Fig. 5 Principal coordinate analysis (PCoA) showing the distribution of *Cicer* RILs and parental lines. Wild *Cicer* ILWC 292 (red, third quadrant) clearly distinguished from cultivated GPF-2 (blue, 1st quadrant) (Color figure online)

by the marker trait analysis where a common SSR marker was found associated with both the traits simultaneously. Considering the key BNF traits, marker CaGM09777 (Ca3) was found to be significantly linked with nodule dry weight and leghaemoglobin content explaining 2.05% and 3.3% variation in nodulation traits (Table 4). Linkage map based on physical distances of the segregating SSR markers associated with key BNF traits on different linkage groups of chickpea is given in Fig. S1.

Discussion

Nitrogen, a key macronutrient determines the productivity of agricultural crops. Its acquisition in legumes confides on two interdependent pathways: first being the uptake of mineral N from soil and second the atmospheric N_2 fixation by the plant–*Rhizobium* association. The contribution of BNF in agricultural systems range from 40 to 70 Tg N y^{-1}, which is approximately 50% of global production of N fertilizers (Goyal et al. 2021). This complex phenomenon of N_2-fixation is an integrated effort of both chickpea host and rhizobia via multiple genes of both the partners. Several microsymbiont genes associated with complex process of BNF have been explored (Moura et al. 2020; Mahumud et al. 2020) however, less emphasis has been placed on the genetic basis for nodulation and BNF efficiency in chickpea due to complexity and relatively large size of plant genome and polygene inheritance of the process.

The wild relative i.e., *C. reticulatum* L. is also an annual species with profound cross-ability with cultivated *Cicer* species and therefore can be utilized to widen the chickpea genetic pool. During the process of crop domestication, multiple genes responsible for key traits are lost which have led to reduced genetic diversity of the crop as well as the associated microbiome over wild parental species. Many wild *Cicer* species have been used in pre-breeding programmes for disease resistance, insecticide tolerance etc. but till date, no systematic efforts have been made to utilize them for improvement of symbiotic efficiency in cultivated chickpea. Further, assessment of genetic variation in chickpea for symbiosis and protein content is also poorly studied. In one of the previous studies by Nagpal et al. (2021), four wild *Cicer* accessions viz., *C. pinnatifidum, C. judiacum, C. bijugum*

Table 3 List of SSR markers co-segregating with key BNF traits at Gurdaspur

Traits	Identified markers	Chromosomal location	Forward sequence	Reverse sequence	R^2	Significance level (%)
Root length	CaGM08648	Ca2	CGCAGGAAAATGGAAGATGT	ATGGGGAACGTTGACAGAAG	0.0322	5
	CaGM 08679	Ca2	TCCTTTTCTCATTCTCAAGCTG	CGTATCCAGTGTCCAACACG	0.0344	
Shoot dry weight (SDW)	CaGM 09901	Ca3	TTTGTTGGTTGAGGTTGTGG	TCACGGAATACATTTCCCCT	0.0231	5
Root dry weight (RDW)	CaGM 09021	Ca2	CACCTAAAATAGGGGGACCAA	GGTACAATGCGTTGGTTTGA	0.0023	5
	CaGM 09227	Ca2	ATGAGAACGTGCTATCGCCT	GGAAGAAATCCTCCTTATTGC	0.0003	
SDW/RDW	CaGM 09021	Ca2	CACCTAAAATAGGGGGACCAA	GGTACAATGCGTTGGTTTGA	0.0273	5
Nodule dry weight	CaGM 08632	Ca2	CACATGACGCAACGAATACC	TCAGTTTCCGCTGCTAACCT	0.0355	1
	CaGM 09021	Ca2	CACCTAAAATAGGGGGACCAA	GGTACAATGCGTTGGTTTGA	0.0165	
	CaGM 09227	Ca2	ATGAGAACGTGCTATCGCCT	GGAAGAAATCCTCCTTATTGC	0.0033	
	CaGM 09214	Ca2	TGCGGTTGTTGAATGACAAT	CCGCCACTCGGATTTAGTTA	0.0107	0.1
Nodule dry wt./ nodule number	CaGM 09021	Ca2	CACCTAAAATAGGGGGACCAA	GGTACAATGCGTTGGTTTGA	0.0055	5
	CaGM 09214	Ca2	TGCGGTTGTTGAATGACAAT	CCGCCACTCGGATTTAGTTA	0.0006	
	CaGM 09227	Ca2	ATGAGAACGTGCTATCGCCT	GGAAGAAATCCTCCTTATTGC	0.0124	
	CaGM 09777	Ca3	CGAACGATGAAAGCACAAAA	TCAGAGGCAGAAGTAAAAGGGA	0.0316	
Total seed protein content	CaGM 08679	Ca2	TCCTTTTCTCATTCTCAAGCTG	CGTATCCAGTGTCCAACACG	0.0272	5
	CaGM 08777	Ca2	AGGGGTCACATGGTTTTGAA	AGGTGGTTGCTGATAGGTGG	0.0433	
	CaGM 08921	Ca2	GCGTGATGAAATTGACATGG	GTGAGACCGTAGGTAGGCCA	0.0288	

**Ca* chromosome

and C. reticulatum were screened for endophytic bacterial diversity and two promising endophytes were identified as potential biofertilizer and bioprotectants in combination with *Mesorhizobium* sp. across different agroclimatic zones of India. This study highlighted the richness of rhizospheric microbiome diversity in wild *Cicer* sp. Further efforts can be aimed to dissect the genetic variation and to map genomic regions for BNF traits in chickpea, which can better explain the role of host plant in the symbiotic association at molecular level. Various cumulative factors viz., soil characteristics, environmental variations, microbial diversity, nodule formation and nitrogenase enzyme activity contribute to BNF efficacy. Sequencing of chickpea genome has presented new resources for crop improvement such as physical chromosomal map and increased number of molecular markers. The importance of crop wild relatives for introgression of desirable traits into breeding material is presently a major research focus.

In present study, significant variation was noticed between the wild and cultivated *Cicer* parents for key BNF traits. The role of environment on BNF was apparent in study and this dependency could be due to the variation in the response of chickpea genotypes to environmental and soil variables. It is evident from the present study that soil moisture and nutrient profiles have direct and indirect effects on BNF efficiency. Yang et al. (2019) documented contrasting traits viz., nodulation and root attributes in two soybean parental lines FC1 and FC2. Under field conditions, FC1 showed shallow root architecture, more roots in top soil and bigger nodules over FC2. Further, the nodulation traits were much more sensitive

Table 4 List of SSR markers co-segregating with key BNF traits at Ludhiana

Traits	Identified markers	Chromosomal location	Forward sequence	Reverse sequence	R^2	Significance level (%)
Plant height	CaGM 02697	Ca1	ACCCCACAATCAGTCCACTC	CACAGGGTTGTAAGGTGGAAA	0.0340	5
	CaGM 09778	Ca3	GAGAGGAGCCAACTGGAACA	TGAACCTGTTGAAGTGAAGCA	0.0544	1
	CaGM 09835	Ca3	TCGACTGTATTGAGGAAAAGTCTC	CAAGGTGTTGGAAAGCACAA	0.0403	
Shoot dry weight	CaGM 00057	Ca1	AGAGGGATTGAGAGGGTGGT	TAATCGAACCCAAAACCCAA	0.0227	5
Nodule number	CaGM 09214	Ca2	TGCGGTTGTTGAATGACAAT	CCGCCACTCGGATTTAGTTA	0.0123	
	CaGM 09227	Ca2	ATGAGAACGTGCTATCGCCT	GGAAGAAATCCTCCTTATTGC	0.0040	
	CaGM 09777	Ca3	CGAACGATGAAAGCACAAAA	TCAGAGGCAGAAGTAAAAGGGA	0.0205	
Nodule dry wt./ nodule number	CaGM 08632	Ca2	CACATGACGCAACGAATACC	TCAGTTTCCGCTGCTAACCT	0.0213	5
Leghaemoglobin content	CaGM 08712	Ca2	GCAAGTCTCACGACATCATCA	AAGCTTGGTTGTGGTGAAGG	0.0185	5
	CaGM 09777	Ca3	CGAACGATGAAAGCACAAAA	TCAGAGGCAGAAGTAAAAGGGA	0.0333	
Grain yield	CaGM 02697	Ca1	ACCCCACAATCAGTCCACTC	CACAGGGTTGTAAGGTGGAAA	0.0314	5
	CaGM 09835	Ca3	TCGACTGTATTGAGGAAAAGTCTC	CAAGGTGTTGGAAAGCACAA	0.0156	

Ca chromosome

to environment changes for FC1 than for FC2, as indicated by different QTLs identified under different environments. In present study also the genomic regions for key BNF traits were different under two agroclimatic zones varying in soil and temperature characteristics. The range of key BNF traits in chickpea RILs exceeded that of the parental lines signifying the possibility of transgressive segregants. Such transgressive segregation suggests that each parent contributed both positive and negative alleles at the loci controlling these traits. The variation in nodulation among chickpea genotypes have also been reported by Mensah and Olukoya, (2007) and Gallani et al. (2005).

Significant positive correlation among key BNF traits and grain yield observed in present study can be attributed to closely linked QTL loci or due to a single QTL with pleiotropic effect. The small but significant correlation coefficients obtained in study reflects the involvement of minor QTLs governing key BNF traits across different agroclimatic zones. The significance level of the correlations might be influenced by the environmental variations. Previous studies (Nicholas et al. 2006; Souza et al. 2000) have documented pleiotropic effect for several morphological and developmental traits in key crops such as common bean and soybean. These interactions are however difficult to confirm owing to the problems associated with distinguishing between multiple strongly linked QTLs that affect each single trait or the single quantitative locus showing pleiotropic effect. To the best of our knowledge this is the first report of the combined study of correlation of all the key traits governing growth, symbiosis and yield in chickpea across agro-climatic zones. In general, it is assumed that traits that have positive correlation move in the same direction during selection, when one is improved, the other also improves positively. In this regard, traits that have strong positive correlation with grain yield are useful in indirect selection for yield especially when they have also high heritability. For negatively associated traits, there should be a compromise between selections for both traits or the breeder should set a minimum standard for one trait while selecting for the other or separate breeding for such traits should be an alternative (Akdemir et al. 2019). For traits with weak association, there may be an independent genetic control between the two traits and improvement in any one of the two would have little effect on the other. Hwang et al. (2014) reported strong positive correlation between nodule number and total nodule weight in *Glycine max.* Similarly, Santos et al. (2013) also reported significant positive and high phenotypic correlation between nodule dry weight (NDW)-nodule number (NN),

NDW-shoot dry weight (SDW), SDW-NN. SDW-NDW/NN, NDW-NDW/NN in soybean.

To minimize the environmental influences on BNF traits, molecular tools are used to observe genetic variation, genetic mapping and molecular breeding. Low proportion of parental polymorphism showed by SSR markers in present study between *C. reticulatum* and *C. arietinum* might be due to narrow genetic base of chickpea. *Cicer reticulatum* is wild progenitor of chickpea and is closely related to cultivated chickpea hence low level of polymorphism was obtained.

The low level of phenotypic variation of key BNF traits explained by identified genomic regions in present study is due to complex BNF process in chickpea. The combined R^2 value explaining the genomic region in each nitrogen fixation component was not high thus highlighting the role of minor QTLs in complex BNF process. In accordance with our results, Santos et al. (2013) mapped two QTLs for SDW (LGs E and L), three for NN (LGs B1, E and I), and one for NDW/NN (LG I); all QTLs were of small effect (R^2-values ranging from 1.7% to 10.0%) in soybean. Fifty-two underlying genes related to BNF (28 nodulins and 24 regulatory genes) were identified in soybean by Schmutz et al. (2010). Similarly, Tanya et al. (2005) reported 19 markers associated with QTLs for nodule number/plant, 16 markers explaining 3.48–19.65% variation in nodule fresh weight/plant, 12 markers linked to QTLs for nodule dry weight per plant and 17 markers associated with plant dry weight in soybean at $P < 0.05$. Souza et al. (2000) used $F_{8:9}$ recombinant inbred lines of common bean and identified QTL explaining 4–11% variation in nodule number.

The identified QTLs with low phenotypic variation in BNF traits in present study can be further fine-mapped by using high-throughput genotyping (SNP genotyping). The wild donor parent i.e., *C. reticulatum* has been extensively used for introgression of disease resistance and other economically important traits in chickpea. Genomic regions identified in present study paved a way to the utilization of this wild progenitor in marker assisted mobilization of biological nitrogen fixation efficiency in elite chickpea genotypes.

Conclusions

Cicer reticulatum represents an excellent reservoir of BNF traits that could be utilized for pre-breeding in chickpea. Segregation of BNF traits in mapping population derived from interspecific cross of chickpea indicates quantitative inheritance of complex BNF process. The markers identified for key BNF traits in present study can be further used for marker assisted selection after validation. The extensive phenotypic data set generated in present study can be helpful in fine mapping for BNF traits with high throughput genotyping. Identified genomic regions can be dissected further for mining the genes governing BNF traits through bioinformatic approaches. To the best of our knowledge this is the first report of identification of genomic regions associated with multiple BNF traits in chickpea.

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Declarations

Authors declare no conflict of interest.

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