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AFLP IN CHICKPEA

Genetic diversity studies of the genus *Cicer* has attracted attraction during the recent past, because it harbors the third most important grain legume crop of the world: chickpea. There is a need to thoroughly assess the level of genetic variability present in chickpea germplasm because its knowledge is a prerequisite for crop improvement programs. Various recently developed molecular techniques including isozyme, Random Amplified Polymorphic DNA (RAPD) and microsatellites have been used for the study of chickpea genome. However, till date there has been no report of using AFLP in the case of chickpea either for genetic diversity studies or for identification of markers. The AFLP technique is a method, by which a selection of restriction fragments of a total genomic digest is detected by amplification using PCR. It is a very versatile method, able to detect the presence of restriction fragments in almost any DNA, regardless of its complexity. The technique allows efficient identification of DNA polymorphisms, because large numbers of restriction fragments may be detected simultaneously. AFLP has been used in many systems to assess genetic diversity, identify markers linked to specific traits and to generate molecular maps. We set out to assess the effectiveness of AFLP in detecting polymorphism in this important legume crop so that it can be used for these useful purposes.

In this study, AFLP reactions were performed to study extent of polymorphism in *Cicer* sp., to study the relationship of *C. arietinum* with their wild relatives *C. reticulatum* and *C. echinospermum* and to derive a phylogenetic relationship between thirty-eight accessions of *C. arietinum* from various eco-geographic regions of the world. (Fig. 5 and Table 1). For each accession, tissue from 5-6 individual plants was pooled and the DNA was isolated. A single pair of enzyme combination (EcoRI + Msel) was used to generate AFLP data. Each DNA was digested, ligated and preamplified using EcoRI (+1) and Msel (+1) primer. Then, thirty selective primer combinations using EcoRI (+3) and Msel (+3) primers were used to generate AFLP profile of these forty accessions. Sequence of oligonucleotide adapters and primers and arbitrary combination numbers used in this study are given in Table 4. Fig. 6 shows a representative sample of each of the thirty primer combination
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electrophoresed with selectively amplified tomato DNA (standard). An AFLP profile of the forty accessions, obtained with primer combination number 9 (E-AAG M-CAA) is shown in Fig. 7. The AFLP reactions were uniform as clearly seen from the nearly equal intensities of respective bands across all the lanes. Only high intensity bands were scored and the reproducibility of the AFLP reactions was checked by randomly duplicating various reactions. Bands in the higher size range were visualized clearly by a longer exposure of the radioactive gel. As seen, some bands marked "M" were found to be monomorphic (present in all the forty accessions). However, polymorphic bands that can clearly distinguish between different accessions were also observed such as bands marked as "P". The monomorphic nature of bands is evident in Fig. 8, which shows the AFLP fingerprint obtained by primer combination number 1 (E-AAC M-CAA).

Relationship between percentage polymorphism and size range of fragments

The occurrence of monomorphic and polymorphic bands produced was studied with respect to their sizes. Fig. 9 shows that out of a total of one hundred and forty-two polymorphic bands obtained using thirty primer pair combinations on the thirty-eight accessions of *C. arietinum*, the maximum (forty-two) lie in the size range of 201-300 bp representing 29.5% of total polymorphic bands while the minimum (six) representing 4.2% of the total polymorphic bands, was in the range of >500. Monomorphic bands showed a nearly linear decrease in their number as the size increased, the maximum (~500) being in the range of 60-100 bp representing 34.4% of total monomorphic bands and the minimum (~75) in being more than 500 bp in size.

Detection of AFLP in the three species of *Cicer*

The genus *Cicer* comprises nine annual and thirty-three perennial species classified into four sections based on their life cycle and morphological and geographical criteria. Of the nine annual species which has been subdivided into four groups, the first group contains the cultigen *C. arietinum* and its presumable ancestors
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*C. reticulatum* and *C. echinospermum*. In this study AFLP has been used to detect polymorphism in these three species of *Cicer*. AFLP reactions were performed on preamplified libraries of thirty-eight accessions of *C. arietinum* and one accession each of *C. reticulatum* and *C. echinospermum*. In all, thirty primer combinations were used. To represent *C. arietinum* as a species, the loci detected by thirty-eight accessions of *C. arietinum* were pooled. Fig. 10 shows that monomorphic and various types of polymorphic bands were detected. Among the polymorphic bands, certain bands were present only in *C. echinospermum* and there were others that were absent only in *C. echinospermum*. Certain loci could be amplified only in *C. reticulatum* only and certain others were distinct because they could not be amplified only in *C. reticulatum* and were present in the other thirty-nine accessions. Further, certain loci could be detected because of their amplification only in the two wild type accessions while there were others that were missing only in the two wild types. All the monomorphic and polymorphic bands were counted manually. A total of one thousand eight hundred and thirty-seven loci were detected in these three species of *Cicer,* of which combination number 1 (E-AAC M-CAA) scanned the maximum loci (ninety-six) and combination number 31 (E-ACC M-CTG) scanned the minimum loci (thirty-seven) (Table 6 and Fig. 11). Of the total loci scanned, 22.8% displayed polymorphism among these three species. The major share (12%) of polymorphism was contributed by *C. echinospermum* followed by *C. arietinum* (5.4%) and *C. reticulatum* (5.2%). Combination number 1 (E-AAC M-CAA) showed the maximum percentage polymorphism (50%) and combination number 40 (E-ACG M-CTT) showed the minimum percentage polymorphism (7.6%). In almost all the primer combinations, the contribution to total percentage polymorphism was highest due to *C. echinospermum*. Among all the primer combinations, the contribution to percentage polymorphism by *C. arietinum* was maximum (12.8%) in the case of combination number 6 (E-AAC M-CTC) (Fig. 12a). Combination number 1 (E-AAC M-CAA) displayed the highest contribution to percentage polymorphism by *C. reticulatum* (14.5%) and *C. echinospermum* (25%) (Fig. 12b and 12c). Among all the primer combinations, the contribution to percentage polymorphism by *C. arietinum* was minimum (0%) in the case of combination numbers 15 (E-AAG M-CTG), 22 (E-ACA
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M-CTC), 32 (E-ACC M-CTT), 39 (E-ACG M-CTG), 43 (E-ACT M-CAG) and 62 (E-AGG M-CTC). Combination number 24 (E-ACA M-CTT) displayed the lowest contribution (0%) to percentage polymorphism by *C. reticulatum* and combination number 54 (E-AGC M-CTC) displayed the lowest contribution (3.3%) to percentage polymorphism by *C. echinospermum*.

**AFLP analysis of relationship between *C. arietinum* and its wild relatives *C. reticulatum* and *C. echinospermum***

The two wild species are known to produce fertile hybrids in crosses with *C. arietinum*, though fertility barriers between *C. arietinum* and *C. echinospermum* do exist. The relationship between *C. arietinum* and its wild relatives was assessed using AFLP.

**Ability of a particular AFLP combination to differentiate between *C. arietinum*, *C. reticulatum* and *C. echinospermum***:

In order to distinguish between the three species it was imperative to assess bands that are specific to *C. arietinum*, *C. reticulatum* and *C. echinospermum*. Fig. 10a, 10b and 10c display such kind of specific bands.

Out of the thirty combinations, combination number 37 (E-ACG M-CTA) produces the maximum number of bands that are specific to *C. arietinum* (11.2%) (Table 7). Combination number 14 (E-AAG M-CTC) produces the maximum number of bands which are specific to *C. reticulatum* (14.2%) and combination number 37 (E-ACG M-CTA) produces the maximum number of bands which are specific to *C. echinospermum* (17.7%).

By assessing the number of distinct bands present in one species and absent in other two, it was observed that the maximum differences (34.5%) among the three species was detected by combination number 14 (E-AAG M-CTC) (Fig. 13). Combination number 38 (E-ACG M-CTC) detects the minimum differences (2.3%) between the three species.
AFLP between any two species of *Cicer*:

The data obtained from AFLP was studied to find out the polymorphism between any two species of *Cicer*. This was done by assessing distinct bands present in one species and absent in other (Fig. 10a, 10b, 10c). In this way each species was compared with the other. The percentage polymorphism of one species with respect to the other was calculated as the percentage of bands present in that particular species over total number of bands detected in both species under consideration. Table 8 shows the results obtained for the three pairs.

1. Polymorphism between *C. arietinum* and *C. reticulatum*:

   Fig. 14a shows the results obtained by comparing polymorphic bands in *C. arietinum* and *C. reticulatum*. It was seen that combination number 1 (E-AAC M-CAA) displays the maximum differences (30.1%) between *C. arietinum* and *C. reticulatum*, the differences being contributed mostly by specific bands present in *C. reticulatum* (22.3%). Combination number 15 (E-AAG M-CTG) displayed the minimum differences between *C. arietinum* and *C. reticulatum* (1.6%). The range of percentage polymorphism for *C. arietinum* was from 0% [(combination numbers 15 (E-AAG M-CTG), 32 (E-ACC M-CTT), 39 (E-ACG M-CTG), 62 (E-AGG M-CTC)] to 18.8% [(combination number 6 (E-AAC M-CTC)]. The range of percentage polymorphism for *C. reticulatum* was from 0% [(combination number 24 (E-ACA M-CTT), 30 (E-ACC M-CTC), 35 (E-ACG M-CAG), 36 (E-ACG M-CAT), 38 (E-ACG M-CTC)] to 22.3% [(combination number 1 (E-AAC M-CAA)].

2. Polymorphism between *C. arietinum* and *C. echinospermum*:

   Fig. 14b shows the results obtained by comparing polymorphic bands in *C. arietinum* and *C. echinospermum*. It was observed that combination number 1 (E-AAC M-CAA) detected the maximum differences (41.8%) in which both *C. arietinum* and *C. echinospermum* contributed equally (20.9% each). Combination number 62 (E-AGG M-CTC) showed minimum differences (5.4%) for these two species. The range
Results of percentage polymorphism for *C. echinospermum* was from 0% [(combination numbers 2 (E-AAC M-CAC), 38 (E-ACG M-CTC), 62 (E-AGG M-CTC)] to 20.9% [(combination number 1 (E-AAC M-CAA)]. The range of percentage polymorphism for *C. arietinum* was from 5.3% [(combination number 54 (E-AGC M-CTC)] to 21% [(combination number 55 (E-AGC M-CTG)].

3. Polymorphism between *C. reticulatum* and *C. echinospermum*:

Fig. 14c shows the results obtained by comparing polymorphic bands in *C. reticulatum* and *C. echinospermum*. It was observed that combination number 1 (E-AAC M-CAA) detected the maximum differences (41.8%), contributed mostly by *C. reticulatum* (26.5%) and combination number 40 (E-ACG M-CTT) showed the minimum differences (7.4%). The range of percentage polymorphism for *C. reticulatum* was from 3.3% [(combination number 11 (E-AAG M-CAG)] to 26.5% [(combination number 1 (E-AAC M-CAA)] and the range of percentage polymorphism for *C. echinospermum* was from 0% [(combination number 62 (E-AGG M-CTC)] to 19.6% [(combination number 37 (E-ACG M-CTA)].

**AFLP analysis of thirty-eight accessions of *Cicer arietinum***

Among the various species of *Cicer*, *C. arietinum* is the only cultivable one. As a result of economic importance of this species, the assessment of genetic diversity within various accessions of *C. arietinum* has been performed using AFLP. Further, the ability of each AFLP primer combination to differentiate between various accessions of *C. arietinum* accessions has been assessed. For this purpose, thirty-eight accessions of *C. arietinum* belonging to various countries were chosen (Table 1).

**Detection of polymorphism within thirty-eight accessions of *C. arietinum***:

In order to find out the number of loci, which can be detected by a particular primer combination, all monomorphic and polymorphic bands (loci) were counted. In all, using thirty primer combinations, a total of one thousand five hundred and sixty
loci were detected in thirty-eight accessions, of which the maximum number of loci (seventy-one) were detected by using combination number 35 (E-ACG M-CAG) and the minimum number of loci (twenty-six) was detected by combination number 31 (E-ACC M-CTG) (Table 9 and Fig. 15a). Of the total loci, 9.1% were polymorphic between these thirty-eight accessions. Combination number 55 (E-AGC M-CTG) displayed maximum percentage polymorphism (21.5%) and combinations number 22 (E-ACA M-CTC) and 32 (E-ACC M-CTT) displayed minimum percentage polymorphism (2.3% each) (Table 9 and Fig. 15b).

Ability of AFLP primer combinations to differentiate between thirty-eight accessions of *Cicer arietinum*:

The presence and absence of bands in a particular accession using a single primer combination produced distinct patterns of bands for the thirty-eight accessions. Based on the number of patterns generated by each primer combination, the ability of each combination to differentiate between these thirty-eight accessions was calculated as shown in Fig. 16. It was observed that combination number 55 (E-AGC M-CTG), which detects 11 polymorphic bands, differentiates between 92.1% (35) accessions (Table 10). Interestingly, combination number 35 (E-ACG M-CAG) which also detects 11 polymorphic bands, was able to differentiate between only 71% (27) accessions whereas combination number 30 (E-ACC M-CTC) which detects 8 polymorphic loci could differentiate between 76.3% (29) accessions. Combination number 55 (E-AGC M-CTG) was not able to differentiate only three accessions numbered 21, 22 and 31. When this combination was used with any of the combination numbers 2 (E-AAC M-CAC), 6 (E-AAC M-CTC), 9 (E-AAG M-CAA), 14 (E-AAG M-CTC), 19 (E-ACA M-CAG), 25 (E-ACC M-CAA), 30 (E-ACC M-CTC), 35 (E-ACG M-CAA), 37 (E-ACG M-CTA), 38 (E-ACG M-CTC), 39 (E-ACG M-CTG), 50 (E-AGC M-CAC) or 57 (E-AGG M-CAA), all thirty-eight accessions could be differentiated.
Distribution and characterization of polymorphic bands among thirty-eight accessions of *C. arietinum*

The AFLP results show that using thirty primer combinations, one hundred and forty-two polymorphic bands were obtained among the thirty-eight accessions of *C. arietinum*. Table 11 provides information about each of these bands including the selective primer extension used to detect it, the size of the band, type of polymorphism (presence/absence), number of accessions in which the band is present/absent and the countries of occurrence. The size of these bands ranged from 75 bp to ~750 bp. These bands distinguished one or more accessions either by their presence (positive polymorphism) or absence (negative polymorphism) in a particular accession. Fig. 17 displays the percentage of various types of polymorphic bands. All forty types of bands ranging from present or absent in one accession to present/absent in twenty accessions were observed. The frequency of individual polymorphic fragments observed among thirty-eight accessions varied from polymorphism present in no accession (0%) to present in half the accessions (50%). All types of polymorphic bands were represented in almost equal percentage over a narrow range of 0 to 7.74%. The 7.74% polymorphic fragments were constituted by band type 35 (present in 35 accessions).

Various types of bands were observed that could qualify as accession specific bands (bands present in only one accession) or that could show closeness between accessions (bands present in two or more accessions). The absence of polymorphic band number 35 (Table 11 and Fig. 18a) and 73 (Fig. 18c) from the Russian accession and presence of band number 102 (Fig. 7) in it might classify them to be considered as accession-specific markers. It was seen that band number 112 is present only in the accession numbers 9, number 14 and number 15 representing three Indian accessions one from Karnataka and two from Maharastra. Band number 139 distinguishes three Indian accessions, among which number 12 is from Madhya Pradesh, number 21 from ICRISAT and number 28 from New Delhi. Band number 1 puts the Indian accession number 33 near *C. reticulatum* as it is present in these two accessions only. One of the band (number 94) is amplified in only two of the Nigerian accessions thus showing
their close relatedness (Fig. 18b). A distinct locus number 53 is observed in the case of accession number 1, which is from Uttar Pradesh, India by virtue of the absence of the amplified fragment in this accession only. Locus number 126 is amplified only in the Indian accessions 9 (Karnataka), 12 (Madhya Pradesh), 14 (Maharashtra), 15 (Maharastra), 21 (ICRISAT) and 33 (Fig. 10b).

Assessment of various AFLP primer combinations for individual accessions of *Cicer arietinum*

In order to determine the efficacy of each primer pair to generate polymorphism in individual accessions, the positive and negative percentage polymorphism generated by each primer pair was plotted against each of the primer combination for every accession separately (Fig. 19). The total amplified products were more or less same for all the accessions since it was primarily driven by monomorphic products that were abundant (~90%). The value of maximum positive and maximum negative percentage polymorphism for each accession and the primer extension producing this percentage polymorphism is depicted in Table 12. Table 13 shows that, for all the thirty-eight accessions, only eight primer combinations are sufficient to detect maximum positive as well as maximum negative percentage polymorphism. Of all these combinations, combination number 14 (E-AAG M-CTC) produces maximum positive percentage polymorphism in fourteen out of thirty-eight accessions which is 36.8% and combination number 55 (E-AGC M-CTG) produces maximum negative percentage polymorphism in seventeen out of thirty-eight accessions (44.7%).

**GENERATION OF PHYLOGENETIC RELATIONSHIP OF THIRTY-EIGHT ACCESSIONS OF *C. ARIETINUM* AND THEIR WILD RELATIVES *C. ECHINOSPERMUM* AND *C. RETICULATUM***

A phylogenetic tree of the thirty-eight accessions of cultivable *C. arietinum* and two of their wild relatives, *C. reticulatum* and *C. echinospermum*, was derived.
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For each primer combination the presence or absence of a polymorphic AFLP band was scored as 1 or 0 respectively across all the forty chickpea accessions. In this way the status of all one hundred and forty-two polymorphic bands was determined. Further, two hundred and eighty-one bands specific to the wild type accessions and one hundred monomorphic bands were also included in the data. These data were used to make pair-wise comparison of all the accessions based on both shared and unique amplification products to generate a similarity matrix using the NTSYS-pc package version 1.5 (Rohlf, 1989).

Generation of dendrogram

The similarity matrix was used to calculate Jaccard’s coefficient of similarity and a dendrogram was created by cluster analysis by the unweighted pair group method on the basis of arithmetic average (UPGMA) using NTSYS-pc package. The resultant dendrogram is shown in Fig. 20. The dendrogram shows that the two wild types are very distinct from the other thirty-eight accessions of cultivable chickpea. The thirty-eight accessions are linked to the *C. echinospermum* at a low similarity coefficient of approximately 0.31 indicating its being the most distant relative of thirty-eight accessions. Further, the other wild relative *C. reticulatum* shows a coefficient of around 0.62 indicating that it is more close to *C. arietinum*. Within the *C. arietinum* genotypes, three distinct groups can be distinguished. Group A comprises of twenty-one genotypes predominantly consisting of six Indian accessions. This group can be divided into three subgroups A1, A2 and A3. Subgroup A1 comprises six accessions out of which four belong to India and one each to Nepal and Uganda. Subgroup A2 comprises of ten accessions among which two each belong to Iraq, Sri Lanka and India. The rest four accessions belong to Algeria, Nigeria, Australia and Pakistan. An accession from Iran is distantly linked to this subgroup. Subgroup A3 comprises of one accession each from Nigeria, Italy, Bulgaria and Iran. The second major group, Group B, comprises of ten accessions out of which four belong to India and one each to Turkey, Sudan, Italy Colombia, Jordan and Australia. Group C, the third major group consists of five accessions, two of which belong to China and one.
each to Ethiopia, Yugoslavia and India (mutant). Accession number 18, from Russia, does not belong to any of these three major groups.

**Principal component analysis**

In order to visualize the dispersion of the individuals in relation to the first two principal axes of variation, principal component analysis was performed. The data was depicted in two dimensions (Fig. 21). As can be seen, there is a clear cut separation of the two wild types *C. echinospermum* and *C. reticulatum* from the rest of the thirty-eight accessions belonging to *C. arietinum* in relation to first two principal axes of variation. The thirty-eight cultivable accessions of *C. arietinum* form a very compact group and are seen to be clustered at a place indicating their very close relationship with each other.

**IDENTIFICATION OF PUTATIVE MOLECULAR MARKERS LINKED TO QUANTITATIVE TRAIT LOCI ASSOCIATED WITH TIME-TO-FLOWERING IN CHICKPEA**

Among the major constraints of chickpea, drought causes about 50% yield loss. The development of short duration varieties that flower early and produce pods before severe drought sets in would help chickpea to escape end-of-season drought and thus increase its yield potential. The timing of the transition to flowering is determined by the interaction of the endogenous developmental competence of a plant with environmental cues that signal the onset of conditions favorable for reproductive success. Many plants respond to changes in day length and extended periods of cold temperatures. The timing of flowering is dependent on the genotype, the seasonal profile, photoperiod and vernalization responses of the plant. The use of genetic approach in *Arabidopsis* has resulted in the identification and cloning of many genes involved in regulating floral transition. Studies indicate that these genes function in different genetic pathways that interact in a complex regulatory network. The nature
and complexity of the pathway indicates the involvement of quantitative trait loci (QTLs) in the fine control of time to flower.

Till date there has been no report of any molecular marker linked to a QTL(s) associated with time-to-flowering in chickpea. Therefore it is imperative to use bulked segregant analysis (BSA) followed by individual plant analysis to identify loci linked to time-to-flowering trait. The principle of BSA lies in the grouping of individuals from lines selected for the trait of interest so that the particular genomic region can be studied against a randomized genetic background of unlinked loci that arise from other non-selected genetic differences between the parents. In this study we have attempted to identify molecular markers linked to QTLs associated with time-to-flowering trait using Amplified Fragment Length Polymorphism (AFLP) and Sequence Tagged Microsatellite Site (STMS) analysis.

AFLP ANALYSIS

Preparation of bulks

Seeds of early flowering chickpea parent ICC5810, late flowering parent Hadas (Table 2) and the early and late flowering progeny lines of F4 and F5 generation of both crosses (Table 3) were grown as described in material and methods. Genomic DNA was isolated from the parent plants and seventy-eight progeny plants. Out of these seventy-eight plants, thirty-nine plants were early flowering and other thirty-nine were late flowering. Each genomic DNA sample was digested with a single pair of enzymes (EcoRI + Msel), ligated to adapters and preamplified using EcoRI (+1) and Msel (+1) primers. In this manner, AFLP libraries of parents and all the seventy-eight individuals were generated. Each preamplified library was quantified and pooled to prepare bulks that represented all the individuals in equal proportions (EB1-EB4, LB1-LB4) (Table 3). In all, eight bulks were made, out of which four were from individuals obtained from cross between Hadas (female) and ICC5810 (male) and the remaining four bulks were from individuals obtained from cross between ICC5810 (female) and Hadas (male). Of the four in each section, two were early
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bulks, one each of F4 and F5 generation, and other two were late bulks, again one each of F4 and F5 generation.

Bulked segregant analysis

Eight EcoRI (+3) and eight Msel (+3) selective primers constituted sixty-four different combinations (Table 4), all of which were used to selectively amplify the ten preamplified libraries (two of parents and eight of bulks). An average of 50 bands was amplified using each primer combination with a total of approximately 3000 loci that were scanned. Of the sixty-four AFLP primer combinations, thirty-seven combinations (57.8%) could not detect any band polymorphic for parents that could also be amplified in the bulked samples (heritable polymorphic loci). The remaining twenty-seven (42.1%) of the AFLP primer pair combinations detected forty-three such polymorphic bands that could also be amplified in the bulked samples. Fig. 22 depicts a representative profile of BSA obtained using primer combination number 6 (E-AAC M-CTC) and number 23 (E-ACA M-CTG). The parental specificity of the bands was judged by the fact that bands specific to early parent were amplified in early parent ICC5810 and all bulks but absent in late parent Hadas. Similarly bands specific to late parent were amplified in late parent Hadas and all bulks but absent in early parent ICC5810. The presence of these polymorphic bands in both late and early bulks suggested a loose linkage with QTL(s) associated with time-to-flowering trait.

Table 14 tabulates the results of bulked segregant AFLP analysis using all twenty-seven primer combinations. The number of polymorphic bands produced by each primer combination varied from one to three. The size range of total bands was from 100 to 700 bp. Of the forty-three polymorphic bands, twenty-eight were early parent-specific and fifteen were late parent-specific. Among early parent-specific bands, the size of the smallest fragment was 102 bp and the largest was of 460 bp. Among late parent-specific bands, the size of the smallest fragment was 99 bp and size of the largest fragment was 342 bp. Among the twenty-seven primer combinations producing forty-three polymorphic bands, primer combination with EcoRI extension AAG detected 45% (9/20) and Msel extension CAA detected 35% (7/20) of the
polymorphic bands. Further, the primer combinations number 9 (E-AAG M-CAA) and 12 (E-AAG M-CAT) produced 3 polymorphic bands each, which is the highest among these primer combinations. The polymorphic bands were designated alphanumeric names such as A9 where "A" stands for AFLP and "9" for arbitrary primer combination number. In case of primer combinations that detected more than one locus, bands were designated as A9a, A9b and A9c respectively depending on the decreasing order of the size of the bands.

**Individual plant analysis**

The twenty-seven primer combinations generating polymorphic bands were used for the AFLP analysis of individual plants that constituted the bulks EB3 and LB3 (Table 3). The recombination frequency of polymorphic loci with the QTL associated with time-to-flowering trait was used as an indicator of the integrity of the loci. Out of forty-three polymorphic AFLP bands, twenty-three bands showed a recombination frequency of value more than 50. The remaining twenty polymorphic bands displayed a recombination frequency of value less than 50 and were chosen for further analysis. Fig. 23-Fig. 28 display the distribution of these twenty early and late parent specific bands in the individual plants after individual plant AFLP analysis. Three primer combinations number 9 (E-AAG M-CAA), number 12 (E-AAG M-CAT) (Fig. 24a, 24c) and number 33 (E-ACG M-CAA) (Fig. 27a) detected certain early parent specific and late parent specific bands that appeared in a mutually exclusive manner indicative of being alleles. Fig. 24a and Fig. 24c display two alleles detected by combination number 9 (A9b and A9c) and number 12 (A12b and A12c). It was observed that two late individual plants (number 6 and number 9) and three early individual plants (number 4, number 11 and number 23) displayed both pairs of alleles indicating their heterozygous nature at these two loci. Fig. 27a displays two alleles detected by combination number 33 (A33a and A33b). It was observed that one early individual plant (number 11) displayed both the alleles indicating its heterozygous nature at this locus. The distribution of polymorphic bands in individual plants is tabulated in Table 15.
Results

STMS ANALYSIS

Preparation of bulks

For preparation of bulks for STMS analysis, ten early and ten late plants from F5 generation of cross *Hadas* x ICC5810 were chosen (EB3 and LB3) (Table 3). The genomic DNA of ten individual early plants was pooled to make early bulk and DNA of ten individual late plants was pooled to make late bulk. The DNA was pooled in such a manner that each individual had an equal representation in respective bulk.

Bulked segregant analysis

Ten primer combinations amplifying STMS sequences (Table 5) were used in a PCR reaction to amplify STMS sequences from these bulks along with the parents *Hadas* and ICC5810. The amplified products were resolved either in a 2% w/v TBE agarose gel or in a 6-12% w/v TBE acrylamide gel. STMS primer combinations amplifying seven loci (TA2, TR29, TS45, TS53, TA64, TS72 and TA125) could detect alleles between the parents. Primer combinations amplifying three loci (TA43, TA80 and TA180) could not detect any polymorphism between parents under these gel conditions. Fig. 29 shows a representative photograph of bulked segregant analysis using primers flanking loci TA2, TR29, TA43, TS45, TS53 and TS72. The pattern of early parent-specific and late parent-specific bands was similar to AFLP bulked segregant analysis. Alleles specific to the early flowering parent were amplified in the early parent and both the early and late bulks but absent in late parent. Similarly, alleles specific to the late flowering parent were amplified in the late parent and the early and late bulks but absent in the early parent.
Individual plant analysis

The seven STMS primer combinations generating polymorphic bands were used to amplify genomic DNA of individual plants that constituted the early and late bulks (EB3 and LB3) (Table 3). Fig. 30-33 display the distribution of early and late parent specific alleles in the individual plants after individual plant STMS analysis. Table 16 tabulates the results of segregant STMS analysis using seven STMS primer combinations. Among the seven primer combinations, four combinations amplifying the loci TA45, TS53, TA64 and TS72 detected alleles that could be separated in a 2% w/v TBE agarose gel. Three primer combinations amplifying the loci TA2, TR29 and TA125 detected alleles that could only be separated in a 12% w/v TBE polyacrylamide gel. In the case of locus TA2 (Fig. 30), two alleles show a difference of ~7 bp indicating the addition of two or three microsatellite repeats in the late flowering parent (Hadas). In the case of loci TR29 (Fig. 31) and TA125 (Fig. 32), it was seen that the difference in size between the two alleles is ~8 bp at locus TR29 and ~7 bp at locus TA125, indicating the addition of two or three microsatellite repeats in the early flowering parent (ICC5810). Further, it was observed that the size difference between the two alleles is approximately 11 bp, 30 bp, 22 bp and 35 bp for loci TS45, TS53, TS64 and TS72 respectively (Fig. 33). This indicates the addition of three to four microsatellite repeats (locus TS45) and ten repeats (locus TS53) in the late flowering parent (Hadas). Similarly, there seems to be an addition of seven microsatellite repeats (locus TA64) and twelve repeats (locus TS72) in the early flowering parent (ICC5810). The polymorphic alleles were assigned alphanumeric names such as S2 where "S" stands for STMS and "2" for locus name. The two alleles detected by each primer combination were designated as “a” and “b” respectively depending on decreasing order of size of fragment.

In the case of locus TA2 (Fig. 30), two of the late individual plants (number 6 and number 9) and two of the early individual plants (number 4 and number 11) show amplification of both alleles indicating their heterozygous nature at this locus. In the case of locus TS72, two of the late individual plants (number 6 and number 9) and two of the early individual plants (number 4 and number 23) show amplification of both
alleles indicating their heterozygous nature at this locus (Fig. 33d). The distribution of these alleles in individual plants using the seven STMS primer combinations is tabulated in Table 17.

RESIDUAL HETEROZYGOSITY AND SEGREGATION DISTORTION OF POLYMORPHIC LOCI

Table 18 shows the genotypic status of each of the twenty individual plants at the twenty-four polymorphic loci (seventeen AFLP and seven STMS) that was determined using the data shown in Table 15 and 17. It was observed that out of twenty individual plants, fifteen plants possessed either the early parent specific locus (E) or the late parent specific locus (L) for each of the twenty-four loci. Five individual plants (number 6, number 9, number 4, number 11 and number 23) showed heterozygosity (H) at different loci. Individual plant number 6, number 9 and number 4 were heterozygous at four loci (A9bc, A12bc, S2ab and S72ab), individual plant number 11 was heterozygous at four loci (A9bc, A12bc, A33ab and S2ab) and individual plant number 23 was heterozygous at three loci (A9bc, A12bc and S72ab). Out of five loci (A9bc, A12bc, A33ab, S2ab and S72ab) four loci (A9bc, A12bc, S2ab and S72ab) showed a higher value of residual heterozygosity than that expected (6.25%) in the F5 generation. It was observed that the value of residual heterozygosity was 25% for A9bc and A12bc, and 20% for S2ab and S72ab. One locus, A33ab, showed a value of 5%, which is less than the expected.

Table 19 shows that the segregation pattern of most of the loci (83.4%) conformed to the expected 1:1 genotypic ratio. Significant deviation from the expected ratio was, however, observed for four loci as shown by chi-square value at one degree of freedom and 5% level of significance. These four loci are A7, A14a, A23a and A33ab and constitute 16.1% of total loci. At three of these loci (A7, A14a and A23a) the early parent allele (E) was more frequent than late parent allele (L). Further, at one locus (A33ab) the late parent allele was more frequent than early parent allele.
ASSOCIATION OF THE POLYMORPHIC LOCI WITH QTL(S) ASSOCIATED WITH TIME-TO-FLOWERING TRAIT

The association of the twenty-four polymorphic loci with QTL(s) associated with time-to-flowering trait was determined by calculating the recombination frequency of the loci with the trait and by performing the Student's t-test for each of the loci.

Recombination frequency (RF) of AFLP and STMS polymorphic loci with QTLs associated with time-to-flowering trait

The recombination frequency of each of the twenty-four polymorphic loci with time-to-flowering trait was calculated using the genotypic status of each individual plant depicted in Table 20. The data represented by heterozygous individuals for specific loci was excluded from the calculation. It was observed that two loci (A33ab and S72ab) have an RF of 31%; two loci (A9bc and A12bc have an RF of 33%; twelve loci (A7, A12a, A14a, A14b, A23a, A23b, A25, A42, A49, S29ab, S45ab and S125ab) have a RF of 35%; one locus (S2ab) has an RF of 37% and seven loci (A6, A9a, A13, A39, A61, S53ab and S64ab) have RF of 40%.

Student's t-test of AFLP and STMS polymorphic loci

In order to establish a statistically significant relationship between the twenty-four polymorphic loci and the time-to-flowering trait, student's t-test was performed using the program Sigma Stat version 3.02 of Jandel Scientific Software (Table 20). The t-test is a parametric test; it is based on estimates of the mean and standard deviation parameters of the normally distributed populations from which the samples were drawn. The data of genotypic status of twenty individual plants for each locus was accessed from Table 18 and the data of mean days to flowering for the family of each individual plant was taken from Table 3. Table 20 shows that in the case of loci A33ab the value of t statistic was 2.25 with 17 degrees of freedom (P = 0.0378).
Results

test results indicate that the difference in the mean value of the two groups in case of locus A33ab is greater than would be expected by chance and that there is a statistically significant difference between the two groups. This analysis shows that the locus A33ab could be a molecular marker linked to a QTL associated with the time-to-flowering trait in chickpea.

GENERATION OF MOLECULAR MARKER MAP OF AFLP AND STMS POLYMORPHIC LOCI

Twelve AFLP and six STMS loci were mapped to five linkage groups (designated LG1-LG5) using the Kosambi function of MAPMAKER ver 3.0 (Lander et al., 1987). The data of heterozygous loci was not included in the input file. The result is shown in Fig. 34. Five AFLP loci (A6, A25, A33ab, A39, and A49) and one STMS locus (S45) showed no linkage with any of the mapped loci and thus could not be mapped. Using a minimum LOD score of 4.0, the eighteen loci covered a span of 27 cM. Linkage Group 1 displayed two clusters of five and four loci each, separated by a distance of 16.6 cM at a LOD score of 1.13. The low value of LOD score indicates that these two clusters could be unlinked. Cluster 1 comprised four AFLP (A7, A9bc, A14a and A23a) and one STMS locus (S72) mapped at the same locus. Cluster 2 comprised three AFLP (A12bc, A13 and A61) and one STMS locus (S2), which were mapped at the same locus. Linkage group 2 comprised three loci A12a, S125 and A42 in which S125 and A42 were mapped at the same locus and separated from A12a by a distance of 2.6 cM at a LOD score of 4.29. Linkage group 2, 3 and 4 comprised two loci each, separated by a distance of 2.6 cM at a LOD score of 4.29. Group 3 comprised A9a and A14b, group 4 comprised S29 and S53 and group 5 comprised A23b and S64.