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# Bulk Segregant Analysis (BSA) and Isolation of Transgressive Segregants for Flowering Time in Field Pea (*Pisum sativum* L.)

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## ABSTRACT

**Background:** Flowering time is an important trait of consideration while developing cultivars with desired crop duration. It is wise to understand the variability for the attribute in the population developed through appropriate parental combination for the desired target trait. For early generation testing and use in marker assisted selection, identifying the molecular markers associated with the trait of interest is crucial to enhance precision and decision making.

**Methods:** An $F_2$ population developed through hybridization of contrasting genotypes for flowering time and maturity *viz*., IPF 4-9 and Khanapur 10 was evaluated with an aim of isolation of superior transgressive segregants and bulk segregant analysis (BSA) was employed for identifying linked molecular markers for flowering time.

**Result:** The wide range of flowering times, spanning from 24 to 90 days was noticed across the genotypes, in addition, substantial variability was recorded for plant height (range), number of pods per plant (range), days to first flowering (range) and seed yield per plant (range). Studies on genetic parameters indicated high genetic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV), along with high heritability and genetic advance for number of pods per axil, number of primary branches per plant, hundred seed weight and number of seeds per pod. Different classes of transgressive segregants based on flowering time with varied maturity were identified. BSA for flowering time using early and late bulks revealed two SSR markers, AB23 and AA206, linked to the trait. The research outcome of isolation of superior transgressive segregants and molecular markers linked to flowering time will be immensely useful and form genetic resource in developing high yielding early genotypes suitable under changing climatic vagaries and for early generation testing and marker assisted selection (MAS).

**Key words:** BSA, Diversity, Field pea, Flowering, Transgressive segregation.

## INTRODUCTION

Pulses, widely recognized as important source of vegetable protein, serve as a staple food for urban and rural populations. There are wide variety of pulse crops cultivated across the countries depending on the suitability to climatic and other edaphic conditions. Among the various pulse crops grown, field pea (*Pisum sativum* L.), often known as dry pea, is an important pulse crop grown in India which is famous for its protein-rich diet. It belongs to the family Fabaceae with genome size of 4.45 gigabases (Kreplak *et al*., 2019). It is also known as garden pea or green pea, when it is produced for the vegetable purpose. It is one of the oldest crops of the world, which was cultivated together with cereals like wheat and barley approximately in 9$^{th}$ millennium BC (McPhee, 2003). It is originated from mediterranean region and has Ethiopia as secondary center of origin. It is a rich source of protein and carbohydrates along with minerals, vitamin A, B and C (Pandita and Pratap, 1990). It has a prominent role in ensuring nutritional diet and plays important role in addressing malnutrition. In India, approximately 22 to 23 million hectares of land with yearly production of nearly thirteen million tonnes is under pulses. India holds sixth position in the production of field pea with the cultivation spread across 6.11 lakh hectares and production of 6.67 lakh tonnes with 1091 kg per ha productivity (IndiaStat, 2024).

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Like several other crops, the productivity of field pea in India is at lower side compared to the global productivity levels due to various reasons including the narrow genetic base and limited utilization of the available variability in improving the local varieties (Kumar *et al*., 2004). Though yield is a complex trait depending on a number of other contributing traits and their association, there is a dire need to enhance the yield potential of the crop (Rao *et al*., 1990). The knowledge regarding the genetic make-up, association between yield and its attributes helps in formulating the selection criteria for improving yield, besides enhancing the scope of success of breeding programme (Sunayana *et al*., 2017).

Genetic variability plays a significant role in crop improvement, as it enables the selection of promising genotypes for enhancing yield coupled with desired quality traits. It also aids in identification of suitable parents for hybridization program (Pratap *et al*., 2024a). Enhancing yield is the most prioritised objective in breeding, however, development of stable high yielding noteworthy cultivars coupled with high photosynthetic efficiency, resistance to biotic and abiotic stresses, early maturity, good initial phases of growth along with high organic matter accumulation potential, high seed protein content with essential amino acids and acceptable ratio between them (Abdou *et al*., 1999; Tiwari *et al*., 2001) is the need of the hour.

In the era of climate change, periodic droughts and hot weather during cropping season are recurrent posing severe yield losses across crops in general and pea is not an exception for this. Crop phenology *i.e*., flowering, podding and maturity plays instrumental role in adaptation of crop cultivars into diverse environments (Berger *et al*., 2006). Earliness is an important trait in crop plants that enables genotypes to complete their life cycle earlier, particularly flowering and maturity, thereby escaping terminal stresses such as drought and heat during later growth stages. This stress-escape mechanism helps in maintaining better grain filling, yield stability and overall productivity under adverse conditions. Consequently, early-maturing genotypes are highly valuable in breeding programs aimed at improving adaptation to stress-prone environments and ensuring sustainable crop production (Jeuffroy *et al*., 2010). In changing climatic conditions, developing early genotypes which can avoid terminal stage drought and heat stresses making them a preferred choice under late sown conditions is in great demand. With the advent of several molecular techniques, it is indispensable to utilise those techniques in breeding programmes for enhanced efficiency, precision and to make highly informed decisions. Bulk segregants analysis (BSA) suggested by Michelmore *et al*. (1991) is a rapid method which involves the bulked DNA of genotypes showing extreme phenotypic values for the target trait in identifying the putatively linked markers to the trait of interest. In view of the above points, the current research was planned to isolate the promising transgressive segregants and to detect the molecular markers associated with the flowering time through BSA.

## MATERIALS AND METHODS

### Evaluation of $F_2$ segregating population for morphological, phenological and productivity traits

The contrasting parents for the target trait, flowering time, were chosen based on two season evaluation. The selected parents, IPF 4-9 and Khanapur 10, are diverse for several other traits (Table 1) besides flowering time. The true $F_1$s were selfed to harvest $F_2$ seeds. In total, 378 $F_2$ seeds were generated to have an optimum population for further studies. To evaluate the phenological, morphological and productivity traits, the experiment was laid out in an un-replicated design in the experimental field of Regional Agricultural Research Station, Vijayapura, Karnataka, India during *rabi* 2022-23 season. Three hundred seventy eight $F_2$ seeds were sown along with parental genotypes. The seeds were planted with 45 cm spacing between rows and 10 cm between plants ensuring single seed per hill in the row length of 5 m. The basal dose of 25 N kg $ha^{-1}$, 60 kg $P_2O_5$ $ha^{-1}$ and 20 kg $K_2O$ $ha^{-1}$ was applied to raise the healthy crop. The prescribed package for cultivation of the crop was followed to establish good crop. Need based plant protection intervention was allowed to control powdery mildew and pod borer incidence. Observations on morpho-phenological and productivity traits were recorded on each $F_2$ plant and the parents.

### Genotyping for Bulk segregants analysis (BSA)

#### DNA isolation

Genomic DNA was extracted from leaves of all the $F_2$ plants and the parents using QIAGEN Plant DNeasy Plant Mini Kit, with the standard protocol mentioned in the manual. After extraction the DNA was stored at -20°C.

#### Qualitative estimation

The quality of DNA samples was verified both on UV-spectrophotometer and also on agarose gel. From the spectrophotometer, absorbance ratio at 260 and 280 nm was recorded and the samples with 1.8 ratio were considered.

#### Normalization of DNA concentration for PCR

Dilution of DNA was done to bring concentration to a relatively equal level (150 ng/µl) by appropriate dilutions with TE buffer. The concentration was estimated by measuring the optical density at 260 nm using an Eppendorf Bio spectrophotometer. The absorbance of the DNA samples was measured at a wavelength of 260 nm against TE buffer blank in a quartz cuvette. 2 µl of DNA aliquot was taken and volume was made up to 1 ml with TE buffer. Optical density (O.D.) was measured at 260 and 280 nm wavelength. Using the relationship of O.D. unit of 1.0 at 260 nm equivalent to 50 µg DNA per ml, the quantity of DNA was estimated by using the following formula:

$$\text{Total quality of DNA (µg/ml)} = \text{O.D. at 260 nm} \times 50 \times \text{Dilution factor}$$

$$\text{Dilution factor} = \frac{\text{Total volume}}{\text{Volume of sample used}}$$

### Molecular markers

To carry out the molecular studies, forty (40) SSR markers were used. The markers were carefully chosen to consider different linkage groups and to cover the genome. Lyophilised primers were synthesised from Sigma-Aldrich with the scale of 0.025 (µmole), later both stock and working solutions were prepared from that.

### Polymerase chain reaction (PCR)

For amplification of the targeted genomic region based on the corresponding markers, the Gradient Eppendorf Thermocycler was employed. The nucleic acid extracted from the leaves of plants, as template DNA along with Taq DNA polymerase, Taq Buffer, dNTPs, $MgCl_2$, forward and reverse primers were used for cyclic amplification of DNA. The reaction mix was prepared fresh depending on the number of samples every time. Each 20 µl mix contained; Taq polymerase: 0.2 µl; Taq Buffer: 2 µl; dNTPs: 2 µl; $MgCl_2$: 1 µl; forward primer: 0.5 µl; reverse primer: 0.5 µl; template: 1 µl; Nuclease free water: 12.8 µl.

### Automatic capillary electrophoresis

The QIAxcel Advanced high through put electrophoresis instrument which includes an array of light-emitting diodes and micro-optical collectors that latch to capillaries within QIAxcel gel cartridges was used to separate the amplicons. The fragments that migrate through a gel matrix within the capillary pass excitation, further, detection of the spots and the signal is transmitted through a photomultiplier tube to the QIAxcel ScreenGel software for data interpretation.

### Parental polymorphic survey

For parental polymorphism, DNA of the parents, IPF-4-9 and Khanapur 10 was extracted and analysed. Forty SSR primers were used to survey the polymorphism. Polymorphic primers were noted. A primer was considered polymorphic, if it amplified different sized bands/amplicons across the parents.

### Bulk segregant analysis for flowering time

Bulk segregant analysis (BSA) was accomplished utilizing the SSR markers having polymorphism among the parental genotypes. Further, DNA bulks of extreme flowering classes *viz*., extreme early and extreme late, were prepared by pooling aliquots containing equal amount of DNA (20 ng/ microliter) from each of the ten early flowering and ten late flowering $F_2$ individuals as suggested by Michelmore *et al*. (1991).

### Statistical analysis

The data on morphological, phenological and productivity traits was analysed using R-studio. The analysis included the determination of mean, range, variance, standard deviation and standard error following standard statistical procedures as described by Singh and Chaudhary (1977). Genetic parameters such as phenotypic, environmental and genotypic variances were estimated to dissect the genetic variability across the genotypes. The coefficients of variation were estimated to dissect the variability (Burton and Devane, 1953), while heritability was determined to comprehend the genetic contribution to phenotypic variation

**Table 1:** Mean with standard error and range of flowering time and maturity of $P_1$, $P_2$, $F_1$ and $F_2$ population.

| Parents | N | Days to 50% flowering | | Days to maturity | |
|---|---|---|---|---|---|
| | | Mean ± SE | Range | Mean±SE | Range |
| IPF-4-9 ($P_1$) | 20 | 60±0.69 | 58-62 | 114±0.76 | 112-115 |
| Khanapur 10 ($P_2$) | 20 | 34±0.53 | 34-36 | 90±0.65 | 89-92 |
| $F_1$ | 20 | 40±0.33 | 36-42 | 100±0.46 | 97-103 |
| $F_2$ | 378 | 59.58±0.78 | 24-90 | 120.87±0.43 | 73-127 |

**Table 2:** Estimates of components of variability, heritability (broad sense), expected genetic advance and genetic advance over mean for ten traits in segregating $F_2$ population of the cross IPF-4-9 × Khanapur 10 in field pea.

| Characters | IPF-4-9 × Khanapur 10 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GCV (%) | GCV category | PCV (%) | PCV category | $h^2$ % | $h^2$ % category | GA (%) | GAM (%) | GAM category |
| Days to first flowering | 8.31 | Low | 8.4 | Low | 98.02 | High | 30.44 | 50.91 | High |
| Days to maturity | 1.72 | Low | 2.17 | Low | 62.59 | High | 10.75 | 8.89 | Low |
| No. of pods per axil | 465.42 | High | 483.10 | High | 92.81 | High | 0.65 | 57.21 | High |
| No. of primary branches per plant | 150.76 | High | 152.59 | High | 97.62 | High | 4.2 | 93.81 | High |
| Plant height | 5.43 | Low | 6.32 | Low | 73.63 | High | 47.99 | 51.89 | High |
| No. of pods per plant | 28.05 | High | 29.69 | High | 89.25 | High | 56.18 | 172.3 | High |
| No. of seeds per plant | 4.39 | Low | 7.68 | Low | 32.76 | Moderate | 89.18 | 68.13 | High |
| Hundred seed weight | 56.91 | High | 62.76 | High | 82.21 | High | 8.45 | 78.08 | High |
| No. of seeds per pod | 136.29 | High | 138.25 | High | 97.18 | High | 2.23 | 57.58 | High |
| Seed yield per plant | 73.23 | High | 75.88 | High | 93.14 | High | 26.44 | 197.7 | High |

(Hanson *et al.,* 1956). Genetic advances and the percentage over mean were also computed (Johnson *et al.,* 1955). QIAxcel screen gel software was engaged for molecular characterization to conduct BSA on flowering time to report the size of amplified fragments.

## RESULTS AND DISCUSSION

### Phenology of parental genotypes, $F_1$ and $F_2$

The genotypes used for deriving the $F_2$ population *viz*., IPF 4-9 and Khanapur 10 were contrasting for flowering time and maturity. The time of flowering of Khanapur 10, an early-flowering genotype was recorded to be 34 to 36 days, while IPF-4-9, a late-flowering one had flowering in around 58 to 60 days. The $F_1$ was observed to be early with 40 days to flower exhibiting dominance of earliness. Wide variability was evident in the $F_2$ population with average flowering time of 59 days and a range between 24 and 90 days. The parents, IPF-4-9 and Khanpur-10, matured in 114 days and 88 days, respectively, while the $F_1$ exhibited a maturity duration of around 100 days (Table 1). Similar results were obtained by Srivastava *et al*. (2025).

### Variability parameters

There was a significant variability for the target trait, flowering time, in the test population. The days to flower initiation ranged between 24 to 90 days with an average of 59.8 days. The trait exhibited low GCV (8.31%) and low PCV (8.4%) with high heritability (98.02%). High GA (30.44%) with high (50.91%) GAM was noticed for the trait. The mean days to maturity was 120.87 falling under the range of 73 to 127 days. Both GCV and PCV were found to be low with 1.72 per cent and 2.17 per cent respectively. Moderate GA (10.75%) with low (8.89%) GAM and high heritability (62.59%) were noticed (Table 2). Similarly, days to first flowering and plant height were reported to exhibit high heritability and genetic advance by Umesh *et al*. (2024). There was a significant variability for yield per plant across the genotypes ranging between 0.11g and 98.04g with a mean of 13.38g. All the genetic parameters *viz*., GCV (73.23%), PCV (75.88%), heritability (93.14%) and GAM (197.7%) were high with moderate (26.44%) GA. The study showed high GCV and PCV for number of pods per axil, number of primary branches per plant, number of pods per plant, hundred seed weight, number of seeds per pod and seed yield per plant. These results are in line with investigations of Singh *et al*. (2012), Khan *et al*. (2017), Pratap *et al*. (2024) and Manimozhi *et al*. (2022).

The observations on heritability and genetic advance for days to flowering, plant height, number of pods per axil, number of primary branches per plant, number of pods per plant, number of seeds per pod, hundred seed weight and

**Table 3:** Frequency of transgressive segregants for morpho-phenological and productivity traits in $F_2$ population of the cross IPF-4-9 × KHANAPUR 10 in field pea.

| Characters | Numbers | Per cent |
|---|---|---|
| Days to first flowering | 193 (07) | 51.06 (3.62) |
| Days to maturity | 337 (08) | 89.15 (2.37) |
| Plant height | 204 (30) | 53.97 (14.7) |
| No. of pods per plant | 334 (64) | 88.36 (19.1) |
| No. of seeds per plant | 307 (36) | 81.22 (11.7) |
| Hundred seed weight | 126 (117) | 33.33 (92.8) |
| No. of seeds per pod | 250 (17) | 66.14 (6.8) |
| Seed yield per plant | 183 (84) | 48.41 (45.9) |

The value in the parentheses indicates superior transgressive segregants in the direction of desirability.

**Table 4:** Promising $F_2$ plants with early flowering and early maturity.

| Plant no. | Days to first flowering | Days to maturity | Seed yield per plant (g) |
|---|---|---|---|
| 34 | 38 | 77 | 19.95 |
| 46 | 40 | 78 | 21.15 |
| 52 | 56 | 111 | 29.87 |
| 132 | 42 | 86 | 35.02 |
| 360 | 59 | 111 | 46.16 |
| 430 | 60 | 106 | 26.11 |
| 472 | 59 | 105 | 20.16 |

**Table 5:** Promising $F_2$ plants with early flowering and late maturity.

| Plant no. | Days to first flowering | Days to maturity | Seed yield per plant (g) |
|---|---|---|---|
| 6 | 47 | 120 | 22.38 |
| 9 | 55 | 122 | 19.18 |
| 19 | 44 | 117 | 19.65 |
| 31 | 41 | 123 | 28.56 |
| 41 | 49 | 124 | 20.81 |
| 60 | 42 | 124 | 26.05 |
| 83 | 59 | 126 | 20.98 |
| 117 | 56 | 120 | 19.76 |
| 135 | 46 | 126 | 18.12 |
| 151 | 55 | 127 | 23.20 |
| 163 | 47 | 125 | 25.87 |
| 182 | 59 | 126 | 35.84 |
| 188 | 55 | 124 | 30.41 |
| 217 | 37 | 124 | 32.30 |
| 239 | 57 | 124 | 33.59 |
| 241 | 58 | 125 | 29.25 |
| 256 | 55 | 123 | 44.20 |
| 269 | 44 | 125 | 43.89 |
| 292 | 58 | 125 | 63.77 |
| 310 | 48 | 125 | 21.70 |
| 364 | 45 | 125 | 25.85 |
| 399 | 58 | 124 | 30.97 |
| 441 | 46 | 123 | 18.53 |
| 456 | 59 | 124 | 38.96 |
| 491 | 54 | 121 | 39.78 |
| 495 | 55 | 127 | 61.99 |
| 498 | 57 | 121 | 64.32 |

seed yield per plant showed high heritability coupled with high genetic advance as reported by Lal *et al*. (2011); Pratap *et al*. (2024); Manimozhi *et al*. (2022); Jaiswal *et al*. (2013) and Jagadeesh *et al*. (2023).

**Isolation of transgressive segregants**

Transgressive segregation is a most desirable phenomenon in breeding wherein the genotypes with outlying phenotypic trait values are isolated. The outliers in the direction of desirability are crucial for morpho-phenological and productivity traits. The transgressive segregants for the phenology were observed based on the trait values. The data on all the genotypes was observed carefully and the number along with per cent transgressive segregants was worked out. For days to maturity, the genotypes outlying the parental values were 337 accounting for 89.15 per cent in both the directions. The screening for transgressive segregants for other traits concurrent with early flowering gave valuable insights and worthy genotypes. Across the genotypes in the population, 250 exhibited transgressive segregation for number of seeds per pod accounting for 66.14 per cent. Plant height showed 204 transgressive segregants which accounted for 53.97 per cent of transgressive segregation. Days to flower initiation was next in order with 193 segregants showing transgressive segregation which accounted to 51.06 per cent (Table 3). The transgressive segregants for phenological traits were categorised into distinct classes *viz*., Early flowering with early maturity; early flowering with late maturity; late flowering with early maturity and late flowering with late maturity with 07, 27, 05 and 60 genotypes respectively (Table 4, 5, 6).

**Table 6:** Promising $F_2$ plants with late flowering and early maturity.

| Plant no. | Days to first flowering | Days to maturity | Seed yield per plant (g) |
|---|---|---|---|
| 50 | 75 | 110 | 24.43 |
| 143 | 68 | 102 | 33.45 |
| 317 | 67 | 107 | 33.03 |
| 347 | 62 | 104 | 34.76 |
| 351 | 63 | 107 | 30.27 |

**Parental polymorphism using SSR markers**

To detect the polymorphism between parents, molecular analysis was carried out using 40 SSR markers to detect the markers which can distinguish the parents, IPF-4-9 (late) and Khanapur 10 (early) genotypically through polymorphic bands. Out of those, twenty-six were polymorphic between the parents indicating high degree of polymorphism of SSR markers and reasonable number of markers for downstream BSA.

**Bulked segregant analysis**

For BSA, the two bulks of the extreme phenotypic values for the phenology (early and late) were used as per Michelmore *et al*. (1991). The bulks prepared by mixing equal quantity of genomic DNA from ten extreme genotypes each were subjected for genotyping using polymorphic markers. Primer pairs that distinguished for the bulks by distinct banding pattern between the early and late bulks for flowering time were carefully observed and recorded (Fig 1).

Among the polymorphic SSR markers (26) between the parents of the cross, two markers *viz*., AB 23 and AA

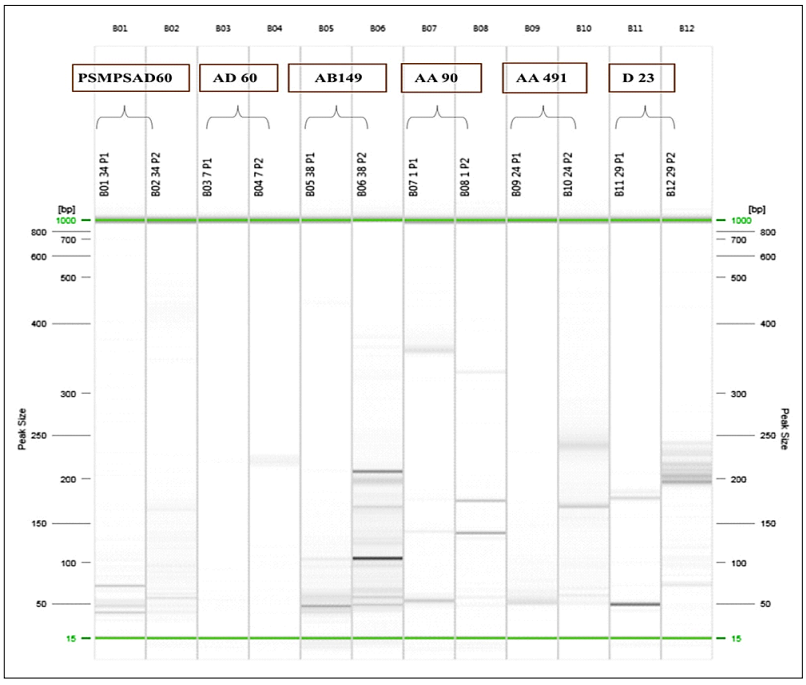


**Fig 1:** Representative image of automatic capillary electrophoresis of SSR markers showing polymorphism between parents IPF-4-9 and Khanpur 10.

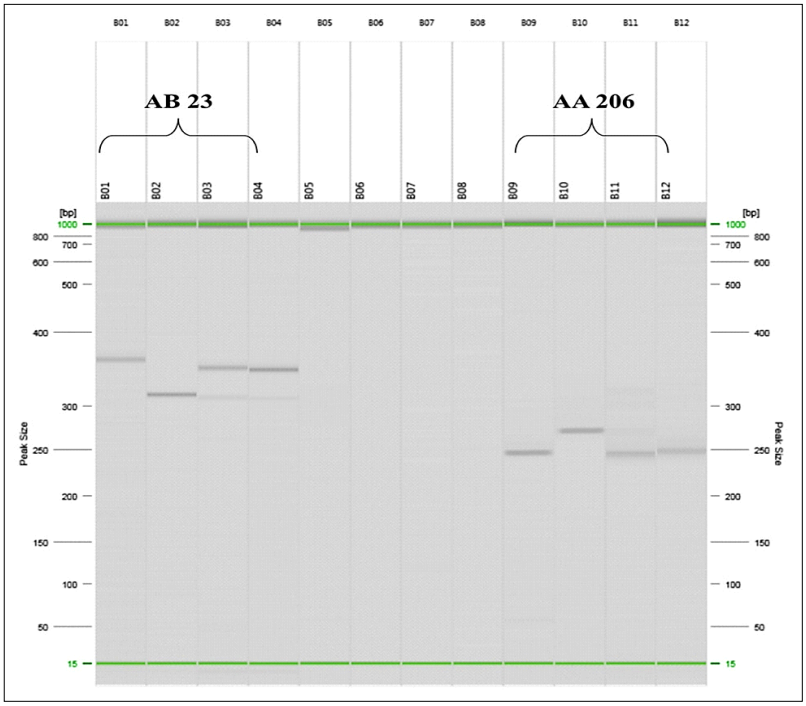


**Fig 2:** Bulk segregant analysis image of automatic capillary electrophoresis with markers AB23 and AA206 showing polymorphism between early and late flowering bulks.

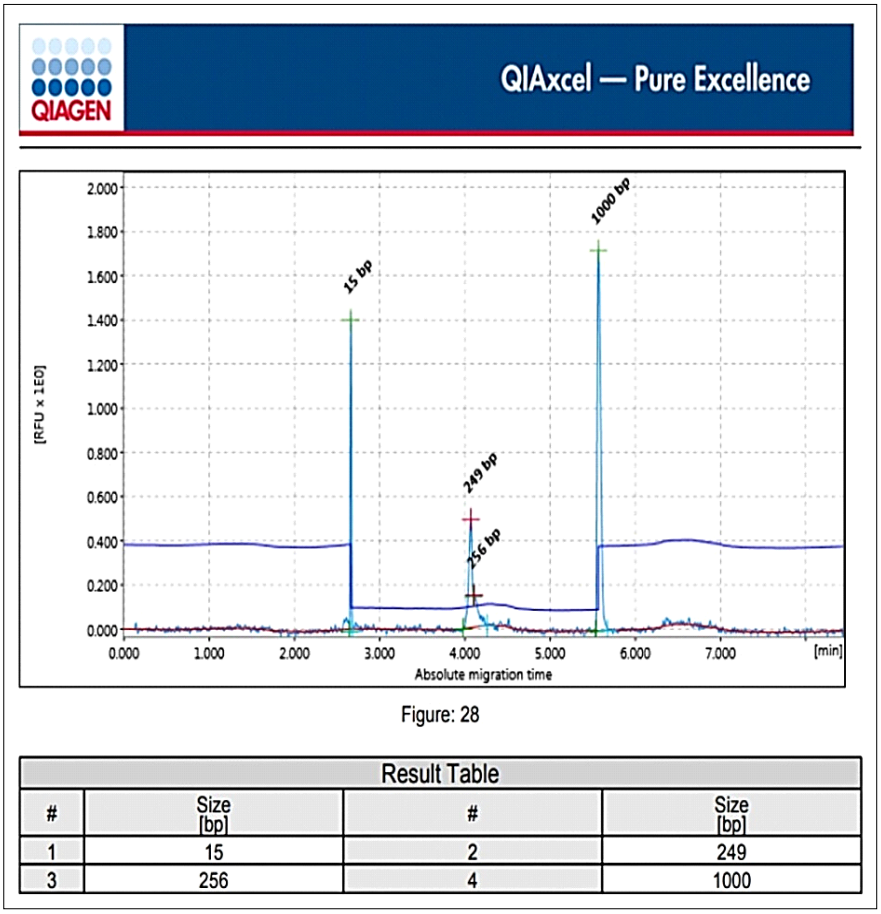


Figure: 28

| Result Table | | | |
|---|---|---|---|
| # | Size [bp] | # | Size [bp] |
| 1 | 15 | 2 | 249 |
| 3 | 256 | 4 | 1000 |

**Fig 3:** Representative result indicating the band size based on migration time.

206 were able to clearly differentiate extreme early and late $F_2$ bulks during BSA analysis (Fig 2 and 3). These two markers revealed the linkage with flowering time and usefulness in selection of early flowering genotypes. The extent of transgressive segregation for the phenology was highly encouraging to isolate desired genotypes in the $F_2$ population, further, detection of two markers associated with the flowering time is noteworthy and highly useful in pea breeding for earliness especially under the changing climatic conditions to avoid terminal stage drought and/ or heat stresses and suitability for late sown conditions. Nunavath *et al*. (2022) reported similar findings using BSA wherein two markers, TA64 and TA142 were reported to be putatively linked to *efl3* locus governing early flowering in chickpea. Other reports on markers linked to important trait include, Uma *et al*. (2016) who reported SSR marker linked to resistance for rust disease in cowpea and Kumar *et al*. (2021) reported SSR markers linked with high Fe and Zn content.

## CONCLUSION

In field pea, it is vital to identify the early genotypes in view of climate vagaries. In this regard, the genetic population developed through the cross between two contrasting parents, IPF-4-9 and Khanapur 10 showcased substantial genetic variability across morphological, phenological and productivity traits. Transgressive segregation for early flowering, early maturity and also for other traits was evident. It was noteworthy that successful identification of the transgressive segregants for earliness coupled with high productivity was possible. Further, the application of bulked segregant analysis, a rapid method to identify the putatively linked markers proved to be efficient and the analysis yielded two markers associated with flowering time. Upon, validation, these markers could potentially serve a significant role in facilitating marker assisted breeding for earliness in field pea.

### Conflict of interest

The authors declare that they have no conflict of interest.

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