

Research Article

Identification and Inheritance of “Cleisto” Genes in Pigeonpea and Their Significance in Producing Genetically Pure Seed

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Abstract

Free filaments of anthers (polyadelphous stamens) and other associated traits such as elongated keel petals wrapped over the wings and the standard considerably delay opening of floral buds, ensuring total self-fertilization in a pigeonpea genotype, ICPL 87154. Inheritance of free filaments and other associated traits of ICPL 87154 were studied in the F₁, F₂, BC₁F₁ and BC₂F₁ generations of its two crosses with high-yielding long-duration pigeonpea varieties, IPA 203 and Bahar. The results suggested that free filaments of anthers in the genotype ICPL 87154 are controlled by double recessive “cleisto” genes, *pct1pct1 pct2pct2*. Backcross derived advance breeding lines with such selfing attributes showed zero per cent natural outcrossing, thus spontaneously sustaining their genetic purity. Two such lines (RCEA 14-1 and RCEA 14-5) with yield potential comparable to the high yielding parent (IPA 203) were selected for further use.

ABBREVIATIONS

DT: Determinate; NDT: Non-Determinate; F₁: First Filial Generation; BC: Backcross Generation; F₂: Second Filial Generation; F₃: Third Filial Generation; RCBD: Randomized Complete Block Design; SAS: Statistical Analysis System

INTRODUCTION

Maintaining the genetic purity of released cultivars, breeding lines, and germplasm is a perennial problem in pigeonpea [*Cajanus cajan* (L.) Millsp.]. This problem was recognized as early as 1908 by pigeonpea breeders at Indian Agricultural Research Institute (IARI), Pusa (Bihar) when they observed a large variation within the progenies of selected single plants [1]. They concluded that this intra-row variability was due to natural cross-pollination affected by a range of flying insects which forage on pigeonpea flowers. This problem persisted for over a century, but no permanent solution was in sight for multiplying genetically pure seed economically. Hence, both the breeders as well as seed growers invariably resorted to the use of physical isolations or insect-proof nets to produce quality seed. Such practices not only add to inefficiencies in breeding and seed management, but also

require additional resources [2,3]. For a long time the pigeonpea breeders were on the lookout for a genetic solution to this perennial constraint. In this context, Saxena et al. [4], selected a floral modification from a cross involving a cultivated variety T 21 and *Atylosia lineata* (now *Cajanus lineata*). During the next year, Saxena et al. [5], identified a similar floral modification, named as “cleistogamous flower” from a segregating population of an inter-specific cross involving *Cajanus albicans* and Pant A2. In the preceding cleistogamous flowers, the flower opening was delayed by 2-3 days, allowing almost total self-pollination. This paper besides describing the floral morphology and inheritance of cleistogamy also reports the extent of outcrossing under natural field conditions. The outcome of breeding efforts made to introgress the “cleisto” genes (cleistogamous trait) in some elite inbred lines through backcrossing is also briefly described.

MATERIALS AND METHODS

Seeds of a determinate (DT) cleistogamous line ICPL 87154 were acquired from International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru (Telangana). In this study, non-determinate (NDT) genotypes (dominant markers) were used as the indicator of cross-pollination on the

DT (recessive) plants of ICPL 87154. During 2009-10 and 2010-11 rainy seasons, ICPL 87154 was grown at the Indian Institute of Pulses Research (IIPR), Kanpur in 100m² plot, and it was surrounded by NDT pigeonpea genotypes as contaminators to determine the extent of natural cross-pollination at this location. In the subsequent seasons of the sowings, counts were made for NDT natural hybrid plants within the population of ICPL 87154. These observations (data not reported) showed no incidence of cross-pollination in ICPL 87154 at Kanpur.

Encouraged with the results, it was decided to study the inheritance of cleistogamous trait and incorporate it into two long-duration popular pigeonpea cultivars Bahar and IPA 203, widely grown in the north-east plains of India (Table 1). These cultivars have normal flowers, and are prone to high natural cross-pollination [3]. These two cultivars were crossed (including reciprocals) with ICPL 87154 for studying its inheritance and to develop high yielding inbred lines with cleistogamous trait through backcrossing using the two cultivars (Bahar and IPA 203) as recurrent parents. The F₁ hybrids were grown during 2011-12. To obtain pure F₂ seed, two plants within each cross were self-pollinated by covering them with nylon net (110 × 90 cm²) with 2 mm mesh size. In addition, four plants within each cross were also used to develop BC₁F₁ populations. In 2012-13 rainy season, the parents, F₁, F₂ and backcross populations were grown with recommended spacing (75 × 25 cm²) and agronomic package and practices. At full flowering stage, in each population counts were made for normal and cleistogamous flower type plants. The segregation data were subjected to the standard chi-square test [6].

To develop inbred lines with cleistogamous flowers, the backcross populations were advanced to produce BC₁F₂ seeds. These BC₁F₂ populations were grown during 2013-14 and selfed to produce BC₁F₃ populations. During 2014-15, the BC₁F₃ populations were grown at the Regional Centre, Darbhanga (Bihar) under ICAR Research Complex for Eastern Region, Patna. From these populations, 20 single plants (each within NDT and DT) with cleistogamous flowers and matching morphological attributes with the recurrent parents were selected at flowering stage, and these were harvested separately. Based on seed yield, only 10 progenies (each within NDT and DT) along with IPA 203 as the control were evaluated for natural out-crossing and productivity during 2015-16.

For studying natural out-crossing, every plant within each entry was examined for the two flower types. For other agronomic traits, data were recorded on ten competitive plants within each plot. Two NDT and one DT backcross derived cleistogamous lines were assessed for productivity during the rainy seasons of 2016-17 and 2017-18 along with leading long-duration varieties (Table 4) in a randomized complete block design (RCBD) at Research Centre, Plandu (Ranchi, Jharkhand). Data were recorded for seed yield (t/ha), number of seeds /pod and 100 seed weight (g). Pooled analysis of data recorded on backcross derived lines and other check varieties during 2016-17 and 2017-18 was performed using Statistical Analysis System [7] available at the Indian NARS Statistical Computing Portal (Indian NARS Statistical Computing Portal; <http://stat.iasri.res.in/sscnarsportal>).

RESULTS AND DISCUSSION

Morphological description of cleistogamous flower

The advance breeding line ICPL 87154 with DT growth habit and cleistogamous flowers was selected from an F₂ population derived from an inter-specific cross between *Cajanus cajan* (L.) Millsp. (cv. T-21) and *Atylosia* (= *Cajanus*) *lineata* W. & A. [4]. In the cleistogamous flower (Figure 1), standard, wings and keels are wrapped with one another; this configuration leads to substantial delay in the opening of flower. In addition, keels of cleistogamous flowers are slightly enlarged, and all the ten stamens are in polyadelphous (free at the base) configuration as compared to normal flower which have diadelphous (9+1) stamen configuration. As all the ten stamens of cleisto flowers are free, they fail to individually push the petals (keels, wings and standard) during the process of opening of flower buds. Moreover, wrapping of petals with one another provide many fold hindrance to foraging honeybees species (eg., *Apis dorsata* and *Apis indica*), which trip to pigeonpea flowers to eat pollen and/or to collect nectar [8]. These features of cleistogamous flowers collectively make themselves highly unattractive to both pollinivore and nectarivore insects, leaving little chance for genetic contamination through insect aided natural outcrossing in pigeonpea.

Inheritance of cleistogamous trait

All the F₁ plants derived from crosses IPA 203 × ICPL 87154 and Bahar × ICPL 87154 displayed complete dominance

Table 1: Salient features of parents used in crossing programme.

Parent/genotype	Pedigree	Salient features
IPA 203	Bahar × Ac 314-314	A non-determinate, long-duration pigeonpea cultivar released during 2015 for cultivation in the north east plains of India. It has normal petals (one standard, two wings and two keels) with diadelphous stamen (9+1) configuration. More than 15% natural outcrossing is normally observed if pollination is not controlled especially when other genotypes are grown in the vicinity.
Bahar	Selection from a landrace of Motihari district in Bihar (India)	A very old variety of long-duration pigeonpea with non-determinate growth habit and compact plant type that is widely cultivated in the north east plains of India. It has petals, stamen configuration and natural outcrossing percentage similar to those of IPA 203. For quality seed production, adequate isolation (at least 200 m) needs to be maintained.
ICPL 87154	T 21 × <i>Atylosialineata</i>	A relatively thermo-sensitive and determinate "cleisto" genotype of medium duration pigeonpea. Standard petal is wrapped over wings and keels, considerably delaying the opening of flower buds. All the 10 stamens are free at the base (non-diadelphous condition). Seed production and maintenance does not need spatial isolation from other genotypes of pigeonpea as it showed zero percent natural outcrossing under Kanpur (Uttar Pradesh, India).

Table 2: Segregation pattern for diadelphous vs. polyadelphous (free) stamens in various generations of two crosses in pigeonpea.

Cross/generation	Normal (diadelphous)	Cleistogamous (polyadelphous)	Expected ratio	Probability
Cross 1 (IPA 203 × ICPL 87154)				
F ₁ (including reciprocals)	18	0	--	--
F ₂	189	14	15:01	P=0.90-0.70
F ₂ (reciprocal)	185	16	15:01	P=0.90-0.70
F ₁ × IPA 203	36	0	--	--
IPA 203 × F ₁	42	0	--	--
F ₁ × ICPL 87154	42	11	3:01	P=0.70-0.50
ICPL 87154 × F ₁	17	7	3:01	P>0.95
F ₁ (reciprocal) × ICPL 87154	38	18	3:01	P=0.30-0.20
Cross 2 (Bahar × ICPL 87154)				
F ₁ (including reciprocals)	27	0	--	--
F ₂	106	11	15:01	P=0.30-0.20
F ₂ (reciprocal)	69	6	15:01	P=0.70
F ₁ × Bahar	65	0	--	--
Bahar × F ₁	20	0	--	--
Bahar × F ₁ (reciprocal)	33	0	--	--
F ₁ × ICPL 87154	15	6	3:01	P=0.95-0.90
ICPL 87154 × F ₁	25	5	3:01	P=0.50-0.30
F ₁ (reciprocal) × ICPL 154	20	8	3:01	P=0.90-0.80
Pooled analysis over two crosses				
F ₁ (including reciprocals)	45	0	--	--
F ₂ (including reciprocals)	549	47	15:01	P=0.20-0.10
BC ₁ (including reciprocals)	196	0	--	--
BC ₂ (including reciprocals)	157	55	3:01	P=0.90-0.80

Table 3: Mean performance of "cleisto" genotypes evaluated over two years (2016-17 & 2017-18).

S.No.	Genotypes	Yield (t/ha)	Seeds/pod (no.)	100 seed wt (g)
1.	RCEA 14-1	1.94	3.58	8.96
2.	RCEA 14-5	2.08	3.77	8.77
3.	RCEA 14-6	1.57	3.67	8.48
4.	IPA 203	1.95	3.85	12.27
5.	Bahar	2.02	4.08	11.03
6.	Type 7	2.33	3.82	11.28
7.	DBGA 7-10	2.10	4.10	13.16
8.	NDA 2	2.20	3.93	12.62
9.	Pusa 9	2.03	3.83	9.94
10.	NDA 1	2.30	4.27	11.10
LSD (P=0.05)		0.18	0.36	0.37

Table 4: Per cent natural outcrossing in promising cleistogamous lines of pigeonpea.

Genotype	Population size*		Off type plants		Per cent outcrossing	
	2015-16	2016-17	2015-16	2016-17	2015-16	2016-17
RCEA 14-1	105	142	1	0	1	0
RCEA 14-5	109	169	0	0	0	0
RCEA 14-6	111	175	0	0	0	0
IPA 203	107	--	17	--	15.88	--

*Plants grown from open-pollinated seeds



Figure 1 A comparison of Normal (left) and cleistogamous (right) flowers.

of normal flower over cleistogamous trait. Plants from the reciprocal crosses also showed the similar results, suggesting the absence of maternal effect on the expression of this trait. In F_2 generation, all the four populations segregated into normal and cleistogamous type plants (Table 2), fitting well to 15 normal: 1 cleistogamous ratio ($P=0.20-0.90$) indicating that the expression of cleistogamous flower was controlled by two recessive alleles. The normal flower was controlled by two duplicate dominant genes.

The backcross progenies of the two cross with ICPL 87154 showed a segregation pattern of 3 normal: 1 cleistogamous ratio ($P=0.20\leq 0.95$). This indicated that the cleistogamous parent (ICPL 87154) was homozygous recessive at both the loci, and its backcrosses led to the observed 3 (diadelphous):1 (polyadelphous) ratio for stamen configuration. The study proved that the modified floral trait was controlled by two recessive genes, and for these the gene symbol $pct1pct1\ pct2pct2$ are assigned. Saxena et al. [4], while deciphering genetics of "partial cleistogamy" in a similar study, reported that free stamen trait was governed by a single recessive gene pct . It appeared that the normal parents (ICPL 1, ICPL 87 and ICPL 85076) used in their study carried only one of the two duplicate genes, and hence the F_1 's were heterozygous at only one locus, leading to identification of only one of the two recessive genes.

The F_2 populations derived from the cross involving the two normal parents (IPA 203 and Bahar) had all normal flowers, implying that both the parents carried the same dominant alleles for this character (normal flower, diadelphous stamens). The backcross progenies with both IPA 203 as well as Bahar had all plants with normal flowers confirming further the role of duplicate dominant alleles for the expression of normal flower. Thus both IPA 203 and Bahar seemed to contain duplicate dominant alleles in homozygous ($Pct1Pct1\ Pct2Pct2$) condition. In the present study, a total of 102 plants with free stamens

(47 in F_2 + 55 in backcross generations) were observed in the segregating generations. However, none of the plants having free stamens (polyadelphous configuration) had normal keel and normal orientation of petals. As the free stamen trait was associated with slightly enlarged keel petals and the standard petal wrapped over wings and keels, the associated traits could be due to "pleiotropic" effects of the same recessive genes.

Productivity of backcross inbred lines

The performance of 20 backcross derived NDT and DT cleistogamous progenies was compared with the check varieties IPA 203 and Bahar for yield and seed size. The best two NDT cleistogamous lines RCEA 14-1 (1790 kg/ha) and RCEA 14-5 (1798 kg/ha) and one DT cleistogamous line RCEA 14-6 (1720 kg/ha) produced similar quantities of grains to that of the best check variety IPA 203 (1810 kg/ha) during 2015-16. These were selected for further evaluation in station trials during 2016-17 and 2017-18. In these trials, the two NDT cleistogamous lines consistently performed at par with the check varieties for yield (Table 3). On the contrary, Choudhary [9] while summarizing his findings on selfing vs. open pollination reported the presence of weak/leaky form of self-incompatibility in pigeonpea, and opined that incorporation of selfing trait (cleistogamous trait) could result in yield penalty in pigeonpea. For seed size, the cleistogamous lines were found segregating, and showed a conspicuous range (7.5 to 11.5 g/100 seeds), indicating opportunity of further selection for enhancing seed size. In addition, some shrivelled seeds were also observed in RCEA 14-1 and RCEA 14-5. Yadav et al. [10], also reported that cleistogamous trait was associated with shrivelled seeds, and stressed the need to break this undesirable linkage to have superior cleistogamous lines with acceptable grain size and productivity.

Out-crossing study

For maintenance of these cleistogamous lines (RCEA 14-1, RCEA 14-5 and RCEA 14-6) and the check variety IPA 203, no isolation was provided. It was interesting to note that these cleistogamous lines (except RCEA 14-1 during 2015-16) showed zero percent natural out-crossing compared to IPA 203 (Table 4). The progeny of individual off type plant selected that appeared in the population of RCEA 14-1 during 2015-16 was grown in the succeeding generation. However, no segregation for floral variation was observed. Therefore, it could be assumed that it might be due to mechanical mixing that occurred during threshing. These three cleistogamous lines are presently maintained through open pollination at the Research Centre, Ranchi. However, other workers have reported 0.16 to 2.67% and 0.14 to 1.33% natural out-crossing in similar cleistogamous lines in India (3 locations) and Sri Lanka (2 locations), respectively compared to the extent of 6.34 to 19.64% in the control genotypes [11].

GENERAL DISCUSSION

Genetic purity is an important access to breeders to achieve high success rate in breeding cultivars and thereafter in multiplying the seed of elite varieties and genetic stocks. In pigeonpea, it is an expensive business due to high rates of genetic contamination caused by uncontrolled insect-aided natural cross-pollination. It occurs at almost every pigeonpea growing

area, but its extent may vary from place to place due to various biological and local ecological factors [8].

Since the pedigree breeding is the prime method to develop new cultivars, selection of phenotypically promising single plants is the key activity. This process may adversely be affected by natural out-crossing in the selected plants because this may produce heterotic natural hybrid plants in the succeeding generations. This will not only influence the breeder's choice of selection in field but also produce unrealistic data, reduce heritability and adversely affect the breeding value of the selections. Besides these difficulties in breeding, the maintenance of genetic purity of commercial seed is very important. If it is not addressed adequately, then released cultivars will lose the key traits for which they were bred. Disease resistance, for example, is one of the key traits in pigeonpea, which is controlled by recessive alleles. It can be safely assumed with an average out-crossing of 20 to 25%, it will take only 1-2 generations of open-pollination to destroy the potential of such cultivars and cause huge losses in the productivity [12].

The logical approach to address this issue is to search a genetic solution. In this context, the incorporation of floral modification reported herein, is the step in the right direction. This trait is rather simply inherited, and its incorporation in pigeonpea breeding materials will be easy. This trait is independent of key yield traits. A weak association reported here with seed size, can easily be taken care of by following appropriate selection schemes. Alternatively, the latest genomics tools can also be used through molecular breeding. Yadav et al. [10], have recently produced a high density molecular map and located genes controlling both cleistogamy as well as seed size in pigeonpea.

CONCLUSION

In conclusion, the cleistogamous trait was able to control the natural out-crossing in pigeonpea. It showed simple digenic (pct1pct1 pct2pct2) inheritance, and its incorporation in breeding materials could be achieved easily. Considering the difficulties faced by breeders and seed producers, it is highly desirable to incorporate this trait through backcross breeding to achieve a permanent solution to the problem of genetic contamination in pigeonpea.

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AUTHORS' CONTRIBUTION

AKC and KBS conceived the idea. KBS provided seeds of cleistogamous line 'ICPL 87154'. AKC and DD developed various generations. DD, AKC and PB conducted trials during respective years at IIPR, Kanpur, RC, Darbhanga and RC, Ranchi, respectively. AKC, DD and PB drafted the MS. AKC and KBS went through the MS, and edited the final version of the MS.

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