Studies on pre-fertilization crossability barriers between cultivated and wild species of pigeonpea [Cajanus cajan (L.) Millspaugh]

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Abstract

The present day commercially grown cultivars of pigeonpea are susceptible to pod borer, pod fly insect pest and a number of diseases those causes huge economic loss every year to the farmers. On the other hand wild relative of cultivated pigeonpea possess genes for insect pest/ diseases resistance besides having some other useful genes. Transfer of desirable gene from wild to cultivated pigeonpea is hindered by several pre fertilization barriers. The identification of pre fertilization barriers will help in the introgression of desirable genes for insect pest and diseases resistance into cultivated types for enhancing and stabilizing pigeonpea production. In present investigation crossability barriers between the cultivated *Cajanus cajan* (PADT-16 and Pusa-992) and wild species *Cajanus scarabaeoides* (ICP-15683, ICP-15703) and *Cajanus platycarpus* (ICP-15663) were examined by pre-fertilization parameters like pollen germination, pollen tube growth, and pollen tube abnormalities. In hand-selfing and intervarietal crosses mean pollen germination and pollen tube growth was higher than interspecific crosses. Strong pre-fertilization barriers were observed in *C. cajan* x *C. platycarpus* crosses. However in interspecific crosses involving *C. platycarpus* as female, low level of reproductive barriers were observed when crossed with *C. scarabeoides* compared to cross with *C. cajan*, though pollen tube reached ovule in both crosses. More pollen tube abnormalities were observed in interspecific crosses involving cultivated and species from tertiary gene pool. Molecular diversity among experimental material through SSR markers exhibited maximum diversity between cultivated *C. cajan* and wild *C. platycarpus* whereas closest relationship was observed between *C.scarabeoides* ICP-15683 and ICP-15703.

Introduction

Pigeonpea (2n = 22) belongs to Kingdom Plantae, Division Magnoliophyta, Class Magnoliopsida, Order Fabales, Family Fabaceae / *Papilionaceae*, Tribe *Phaseoleae* and Genus *Cajanus*. In the sub-tribe *Cajaninae*, only one species, *Cajanus cajan* has been domesticated and is being cultivated. It ranks sixth in global grain legume production, and worldwide it is cultivated in about 6.99 m ha area with total production of about 5.93 m tones annually with average productivity of 852 kg/ha (FAOSTAT, 2018). Pigeonpea crop is widely grown in South Asian countries including India, Pakistan, Sri Lanka, Nepal, Bhutan, Bangladesh and Myanmar, in African countries *viz*. Uganda, Ghana, Zimbawabe, Zambia and Mozambique and also in some caribbean countries. Globally, India is the largest producer and consumer of pigeonpea with an area of 4.4 m ha, annual production of 3.68 million tons and productivity of 832 kg/ha (Anonymous, 2019). India contributed about 72% of global pigeonpea production (Sharma et al. 2019). In pigeonpea, to diversify and broaden the genetic base of cultivated germplasm, pre breeding efforts are urgently required involving mainly those wild species that carry useful alien genes for improving seed yield, quality and resistance to several biotic and abiotic stresses that affects the crop adversely. Wild species are rich reservoir of useful alien genes those are no longer available within the cultivated gene pools (Doyle, 1988; Tanksley and McCouch, 1977). In case of pulses including pigeonpea, occurrence of useful gene is more frequent in the secondary and tertiary gene pool (Mallikarjuna et al. 2006). Evaluation of wild species of secondary and tertiary gene pool in pigeonpea has shown many desirable characteristics like early maturity, prolific podding, high protein content, resistance to diseases like wilt, *Sterility mosaic*, *Phytophthora* stem blight and resistance to pod borer (*Helicoverpa armigera*) and spotted pod borer (*Maruca vitrata*) insect pest that can be introgreseed into cultivated species to make them more adaptive and productive (Saxena et al. 1990; Mallikarjuna et al. 2006; Bohra et al. 2015).

The extent of croassability of wild species with cultivated *C. cajan* largely depends on sporophytic compatibility supporting successful fertilization and gametophytic compatibility as expressed in karyomorphlogy and chromosome pairing during meiosis. To exploit the variability present in the gene pool or germplasm, the primary requirement is to have a sound understanding of croassability barriers those prevent the gene flow between wild and cultivated species and these may be at pre-fertilization as well as post- fertilization stages (Stebbins, 1958). Pre-fertilization barriers mostly include non-germination of pollen grains on the stigmatic surface, delayed pollen tube growth along with other structural abnormalities like twisting, swelling, bi-furcated tip, bursting of pollen tubes midway in style, coiling and twisting of pollen tubes in the style and pollen tubes may cease growth before reaching the ovary or may not be guided to the micropyle and embryo sac after reaching the ovary (Stalker, 1980; Ram et al. 2007).The analysis of genetic diversity is an important factor for the breeding of highly productive cultivars in crop improvement programmes. Molecular markers serve as important tools to study the genetic diversity. Molecular analysis in pigeonpea revealed that in cultivated pigeonpea, molecular variation is much less as compared to morphological variation, however, in the wild species of *Cajanus* there is a high degree of molecular variation as compared to morphological variation (Yang et al.2006). Simple Sequence Repeat (SSR) markers are PCR based markers that are polymorphic, co-dominant, reliable, reproducible and abundant throughout the genome and have been used in assessment of genetic diversity in pigeonpea. SSRs are being effectively used by several workers to analyses genetic diversity in pigeonpea (Sahu et al. 2016; Sharma et al. 2020). To identify the pre fertilization barriers among the cultivated and wild pigeonpea, the present investigation was conducted to understand the exact nature of pre-fertilization barriers and molecular genetic diversity between different species so that depending on type of barriers effective techniques can be developed to overcome these barriers for introgression the desirable gene from wild to cultivated types. The results of present study will help in developing varieties with enhanced productivity and resistance to important diseases and insect pests .

Material And Methods

Experimental site and Plant material

The present investigation was carried out at Norman E. Borlaug Crop Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, India during *kharif* 2019 crop season. The experimental material used in the present study comprised of two cultivated varieties of *Cajanus cajan viz*. PADT-16 (Determinate type) and Pusa-992 (Indeterminate type), two accessions of *Cajanus scarabaeoides viz*. ICP-15683 and ICP-15703 belonging to secondary gene pool having desirable traits like prolific podding, more number of seeds per pod, early maturity and resistance to pod borers (*Helicoverpa armigera*) pest and one accession of *Cajanus platycarpus viz*. ICP-15663 belonging to tertiary gene pool having desirable traits like extra early flowering and maturity, photoperiod insensitivity, annuality, resistance to *Phytophthora* stem blight, *sterility mosaic* disease, pod borer and pod fly insect pest. The seeds of the wild type were obtained from Indian Institute of Pulses Research, Kanpur. Staggered sowing was done to synchronize the flowering time of different species. Since wild species have poor germination, therefore to ensure good germination; scarification of the seeds was done using a sharp knife and thereafter scarified seeds

were sown in the pots in glasshouse. Five seeds were sown in each pot. The seeds of cultivated varieties (PADT-16 and PUSA-992) have good germination and hence were not scarified.

Interspecific hybridization and pre fertilization studies

The seeds of cultivated and wild types were sown in glasshouse during *kharif* season of 2019. For study emasculation of flowers was carried out in the morning hours in between 6:00 a.m. to 8:00 a.m. The pollen was collected from dehiscing anthers of freshly opened flowers of male parents. The emasculated flower was opened carefully and the pollen mass was placed on the stigma without harming the stigmatic surface and the style. In hand selfing, flower bud was emasculated and subsequently it was pollinated with pollens from other flower of the same plant. The hand selfing, intervarietal and interspecific crosses attempted in the present investigation are listed in Table 1. The pollinated flower buds were detached from the plants at different time intervals *viz.* 1, 8, 24, 48 and 60 hours after pollination (HAP). For each time interval 10 pollinated flower buds were collected and used for microscopic studies. These flower buds were fixed in freshly prepared solution of 1:3 aceto-alcohol (glacial acetic acid: 70% ethanol) for 24 hours and thereafter transferred in 70% ethanol and stored at 4^{o}C in the refrigerator till further use. To identify the crossability barriers, parameters such as pollen germination percent, pollen tube growth, and pollen abnormalities were studied using bright-field and fluorescent microscopy. The microscopy works were carried out in Cytogenetics and Molecular Marker Laboratory of the Department of Genetics and Plant Breeding.

Table 1
The percent pollen germination among self, intervarietal and interspecific crosses of pigeonpea

Self/cross combinations	Nature of cross	Pollen germination (%)											
		1HAP		8HAP		24HAP		48HAP		60HAP		Mean	
PADT-16 x PADT16	Selfing	25.82		63.56		73.35		79.12		80.82		64.53	
PUSA-992 x PUSA992	Selfing	28.20		66.89		76.72		81.50		85.43		67.74	
ICP-15683 x ICP-15683	Selfing	22.76		56.24		68.4		74.85		77.14		59.87	
ICP-15703 x ICP-15703	Selfing	20.66		50.8		61.74		69.92		73.80		55.38	
ICP-15663 x ICP-15663	Selfing	17.85		41.62		54.25		59.58		62.86		47.23	
Grand Mean of selfed												58.95	
		Direct crosses						Reciprocal crosses					
		1HAP	8HAP	24HAP	48HAP	60HAP	Mean	1HAP	8HAP	24HAP	48HAP	60HAP	Mean
PADT-16 x PUSA-992	Intervarietal	24.00	60.20	68.42	74.25	77.5	60.87	22.45	52.50	57.45	67.00	72.65	54.41
ICP-15683 x ICP-15703	Intervarietal	17.88	44.60	52.00	58.75	65.5	47.74	18.70	47.00	56.85	66.52	70.58	51.93
Grand Mean of intervarietal direct crosses							54.305						53.17
PADT-16 x ICP-15683	Interspecific	11.76	19.50	26.86	31.50	38.67	25.65	17.50	23.86	30.25	39.20	50.28	32.21
PADT-16 x ICP-15703	Interspecific	8.68	14.38	19.18	29.04	32.12	20.68	8.78	19.80	22.30	35.85	41.60	25.66
PADT-16 x ICP-15663	Interspecific	7.92	13.20	17.45	22.36	26.80	17.54	8.42	15.74	19.38	28.75	30.28	20.51
PUSA-992 x ICP-15683	Interspecific	13.72	24.64	30.88	39.75	45.82	30.96	14.64	35.72	47.75	52.60	58.35	41.81
PUSA-992 x ICP-15703	Interspecific	6.50	18.83	22.50	32.65	35.56	23.20	12.50	26.10	33.65	42.55	46.35	32.23
PUSA-992 x ICP-15663	Interspecific	5.23	9.25	16.42	18.00	20.85	13.95	13.50	21.65	25.00	33.60	39.35	26.62
ICP-15683 x ICP-15663	Interspecific	5.86	9.48	13.48	16.30	22.15	13.45	9.85	16.80	21.50	34.50	46.70	25.87
ICP-15703 x ICP-15663	Interspecific	7.55	12.65	22.72	31.18	36.48	22.11	15.10	24.68	37.25	49.66	55.38	36.41
Grand Mean of interspecific crosses							20.94						30.17

Bright-field microscopy

Out of ten flower buds preserved in each cross, five buds were used for bright field microscopy. The fixed flower buds were rinsed gently in distilled water. Pistils were gently removed with the help of forceps, and then treated with 1N HCl for 15–20 minutes. These pistils were again rinsed with distilled water to remove any traces of HCl and then stained with 1% aniline blue, staining was done for 2–3 minutes. Aniline blue was prepared by dissolving 1 gram of aniline blue in 100 ml of lactophenol, a 1:1:1:1 mixture of lactic acid, phenol, glycerol and distilled water (Gerlac, 1969; D'Souza, 1972). After staining, the pistils were destained in destaining solution (40% acetic acid, orthophosphoric acid and distilled water in 1:1:1 proportion), until all the stain was removed from the stylar tissue. The pistils were again washed with distilled water to remove traces of destaining solution. The pistils were placed on glass slide and mounted in pure lactic acid. Covered carefully with cover slip, to avoid bubble formation and slight pressure was applied using back of dissecting needle to spread the stigmatic surface. The slides were observed under bright-field microscope, pollen grains and pollen tubes took deep blue colour while stylar tissue at cut end was lightly stained. Pollen germination and abnormalities percent was measured. Photomicrographs were taken using camera inbuilt LEICA DM 6000B microscope.

Fluorescent microscopy

The remaining five pollinated buds were used for fluroscence microscopy to observe pollen germination, pollen tube growth and pollen tube abnormalities. The preserved buds were taken out and washed with distilled water. Pistils were carefully separated from the flowers with the help of forceps. Presence of abundance of hair on ovary and lower style region in *C. platycarpus* and *C. scarabaeoides*, created hindrance in observing the growth of pollen tubes and its abnormalities, if any. Therefore, scrapping of hairs from ovary region was done and after that small transverse section slit was made for observing pollen tubes growth and its entry in ovule. Thereafter the pistils were treated with 8N NaOH for 2–4 hours for clearing and softening the tissue. The pistils were washed with distilled water to remove traces of NaOH. After that pistils were transferred to Decolorized Aniline Blue (DAB) stain for 30 minutes to 1 hour. Decolorized Aniline Blue was prepared by dissolving 0.1% water soluble aniline blue dye in 0.1 M Phosphate buffer and the pH was adjusted to 10. The pistils were mounted in glycerol and DAB. The slides were kept in dark until observed under microscope (LEICA DM6000B).

Observations Recorded

(i). *Pollen germination*

The pollen grains which had pollen tube longer than its diameter were considered as germinated. At different intervals pollen germination was calculated as,

Percent pollen germination = $\frac{Numberofgerminatedpollengrainonstigma}{Totalnumberofpollengrainsonthestigma}$ x 100

(ii). *Pollen tube growth*

The growth of pollen tube length was measured using inbuilt micrometry of LEICA DM6000B fluorescence microscope.

(iii). *Abnormal pollen tubes*

Those pollen grains forming more than one pollen tube or the pollen tubes which grew in the wrong direction or had forked, or pollen tubes got twisted or got bursted, or swollen at the tip were considered as abnormal and the percentage of these abnormal pollen tubes was calculated as

Percent abnormal pollen tubes = $\frac{TotalNumberofabnormalpollentubes}{Totalnumberofpollentubes}$ x 100

Photomicrographs were taken with the help of LEICA DM6000B fluorescence microscope at the magnification of 100X, 200X and 400X.

Molecular genetic diversity analysis

The molecular work was carried in the Pulse Breeding lab of the Department of Genetics and Plant Breeding, GBPUA&T, Pantnagar. Thirty microsatellite markers distributed over the whole genome were used in present study and were selected from the published source (Dutta et al. 2011; Bohra et al. 2011). The primers were synthesized from Bangaluru GeNei Ltd. Assay Buffer, Taq DNA polymerase and dNTPs were purchased from Bangaluru GeNei Ltd., Bengaluru, India. The SSR markers used in the present study are listed in Supplementary Table 1.The genomic DNA was extracted for molecular characterization studies by using the Cetyl tri- methyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with very slight modifications. A single PCR reaction contains a total volume of 10.0 µl and consisted of 1.0 µl DNA template (100 ng/µl), 1.5 µl Taq buffer (10X) with 15mM $MgCl_2$, 0.3 µl dNTPs 10 mM, 0.2 µl Taq polymerase 3U/µl and 1.0 µl primers (50 ng) and 6 µl part is ddH_2O were used for each PCR reaction. The amplification reaction was carried out in thermocycler, Peq STAR, PEQ LAB Ltd., Eppendorf Ltd., Germany with a program for an initial denaturation at 94 degree Celsius for five minutes followed by 35 cycles of 94 degree Celsius for one minute, annealing temperature vary with primer (50–65 degree Celsius) for 1 minute, elongation 72 degree Celsius for 1 minute and a final extension at 72 degree Celsius for 8 minute. Horizontal gel electrophoresis unit was used for fractionating PCR products on agarose el (3.0 %) and EtBr for visualization of bands under UV Alpha-imager.

Molecular marker data analysis

The amplification data generated by SSR markers was analysed using SIMQUAL SCS program of NTSYS-PC software v. 2.02 (Rohlf, 1998) to generate Jaccard's similarity coefficient (Jaccard, 1908). These similarity coefficients was used to construct a dendrogram depicting genetic relationships among the cultivars by employing the unweighted pair group method of arithmetic averages (UPGMA) algorithm and SAHN (sequential, agglomerative, hierarchical and non-overlapping) clustering (Sneath and Sokal, 1973).

Results

Pollen Germination percent

A close perusals of Table 1 indicated that pollen germination in self-crosses, direct intervarietal and interspecific and in respective reciprocal crosses was minimum at 1 hour after pollination (HAP). Pollen germination increased gradually and was recorded maximum at 60 HAP in all the selfed, direct and reciprocal crosses. The pollen germination in self of Pusa-992 and PADT-16 are presented in Fig. 1 (a & b). The maximum pollen germination was recorded among all the selfed crosses in the primary gene pool species indeterminate cultivated variety Pusa-992 at 60 HAP (85.43%) and minimum was recorded in the selfed crosses of tertiary gene pool species *C. platycarpus* (17.85%) at 1 HAP. It was further observed that at 60 HAP, maximum pollen germination was recorded in primary gene pool species cultivated types indeterminate variety Pusa-992 (85.43%), which was followed by determinate variety PADT-16 (80.82%), secondary gene pool species *C. scarabaeoides* ICP-15683 (59.87%), ICP-15703 (55.38%) and was recorded lowest in the selfed crosses of tertiary gene pool species *C. platycarpus* ICP-15663 (47.23%). Among the intervarietal crosses, the pollen germination increased steadily and was maximum at 60 HAP in direct crosses between both cultivated types (77.50%) and between both wild *C. scarabaeoides* species (65.50%). similar trend was recorded for the reciprocal intervarietal crosses. It was further recorded that the pollen germination was more at all the time intervals *i.e.* 1, 8, 24, 48 and 60 HAP in the direct and reciprocal crosses involving cultivated primary gene pool species PADT-16 and Pusa-992 as compared to the direct and reciprocal crosses involving secondary gene pool species *C. scarabaeoides* ICP-15683 and ICP-15703. Among all the interspecific crosses, the maximum pollen germination percentage was recorded in the cross between cultivated Pusa-992 and secondary gene pool species *C. scarabaeoides* ICP-15683 (45.82%) at 60 HAP. It was also observed that among all the reciprocal interspecific crosses, the maximum pollen germination percentage were recorded involving the same parents (41.81%) at 60 HAP. The minimum pollen germination among all the direct crosses at 60 HAP (20.85%) was recorded in the interspecific cross between cultivated indeterminate genotype Pusa-992, a primary gene pool species and wild *C. platycarpus* ICP-15663, a species of tertiary gene pool. However, among reciprocal interspecific crosses at 60 HAP, the minimum pollen germination percentage was observed in the cross ICP-15663 x PADT-16 (20.51%). In the direct interspecific crosses involving cultivated determinate genotype PADT-16, the maximum pollen germination was observed at 60 HAP and it was highest in the cross PADT-16 x ICP-15683 (38.67%), followed by the cross PADT-16 x ICP-15703 (32.12%) and PADT-16 x ICP-15663 (26.80%). In the reciprocal interspecific crosses involving cultivated genotype PADT-16, the highest pollen germination was recorded at 60 HAP and it was highest in the reciprocal cross ICP-15683 x PADT-16 (50.28%), followed by ICP-15703 x PADT-16 (41.60%) and ICP-15663 x PADT-16 (30.28). In the direct interspecific crosses involving cultivated indeterminate genotype Pusa-992, the maximum pollen germination was observed at 60 HAP and it was highest in the cross Pusa-992 x ICP-15683 (45.82%), which was followed by the cross Pusa-992 x ICP-15703 (35.56%) and Pusa-992 x ICP-15663 (20.85%). In the reciprocal interspecific crosses involving cultivated indeterminate genotype Pusa-992, the highest pollen germination was recorded at 60 HAP and it was maximum in the reciprocal cross ICP-15683 x Pusa-992 (58.35%), followed by ICP-15703 x Pusa-992 (46.35%) and ICP-15663 x Pusa-992 (39.35%). In the interspecific crosses involving the species of secondary and tertiary gene pool, the highest pollen germination was recorded at 60 HAP in the cross between *C. scarabaeoides* ICP-15703 x *C. platycarpus* ICP-15663 (36.48%) among both the direct crosses. In the reciprocal interspecific crosses highest pollen germination was observed at 60 HAP in the cross involving same parents *i.e.* ICP-15663 x ICP-15703 (55.38%). It was interesting to note that in all the interspecific crosses the pollen germination percentage was highest at 60 HAP, however, pollen germination was significantly more in reciprocal crosses as compared to the respective direct crosses. It was further observed that in interspecific crosses when the species from higher gene pool were used as female and from lower gene pool as pollen parents, the pollen germination was more. In case of crosses involving primary x secondary gene pool more germination was recorded when genotype from secondary gene pool was used as female parent and primary was used as pollen source. Similar results were also obtained in case of primary x tertiary and secondary x tertiary gene pool crosses. On the basis of these findings this can be concluded that the species of secondary and tertiary gene pool may be used as female parent in interspecific crosses for increasing the chances of getting interspecific hybrids.

Pollen Tube Growth

In general, the pollen tube length increased with the increased time and was observed to be longest at 60 HAP in self, direct and reciprocal intervarietal crosses and direct and reciprocal interspecific crosses (Table 2). It is evident from the Table 2 that among all the self crosses, the longest pollen tube at 60 HAP was observed in selfed cultivated indeterminate variety Pusa-992 (3715.26 µm) followed by another cultivated determinate variety PADT-16 (3582.42 µm), tertiary gene pool genotype *C. platycarpus* ICP-15663 (3196.15 µm), secondary gene pool genotype *C. scarabaeoides* ICP-15703 (2625.66 µm) and minimum pollen tube length at 60 HAP was recorded in *C. scarabaeoides* ICP-15683 (2597.58 µm). Pollen tube grew longest at 60 HAP in the direct (3475.42 µm) as well as in reciprocal crosses (3380.48 µm) involving both the cultivated varieties as compared to the intervarietal direct (2805.60 µm) as well as reciprocal cross (2663.75 µm) among secondary gene pool genotype *C. scarabaeoides* ICP-15683 and ICP-15703. Among the direct interspecific crosses, the maximum pollen tube length at 60 HAP was observed in the cross PADT-16 x ICP-15703 (3427.94 µm), followed by the cross Pusa-992 x ICP- 15683 (3402.65 µm) and Pusa-992 x ICP-15703 (3376.48 µm). The least pollen tube length was recorded in the cross between *C. scarabaeoides* ICP-15683 x *C. platycarpus* ICP-15663 (1952.38 µm). However, among the reciprocal interspecific crosses, the maximum pollen tube length at 60 HAP was recorded in the cross *C. platycarpus* ICP-15663 x cultivated PADT-16 (3302.5 µm) followed by the reciprocal cross ICP-15683 x ICP-15663 (3248.72 µm), ICP-15663 x Pusa-992 (3118.35 µm) and the smallest pollen tube in reciprocal interspecific crosses was observed in ICP-15703 x Pusa-992 (2513.24 µm).

Table 2
The pollen tube length among self, intervarietal and interspecific crosses of pigeonpea

Self/cross combinations	Nature of cross	Pollen tube growth (µm)										
		1HAP	8HAP	24HAP	48HAP	60HAP	Mean					
PADT-16 x PADT16	Selfing	2208.15	3539.55	3550.46	3563.38	3582.42	3288.79					
PUSA-992 x PUSA992	Selfing	2433.55	3675.38	3682.62	3689.84	3715.26	3439.33					
ICP-15683 x ICP-15683	Selfing	1580.42	2555.62	2570.33	2583.67	2597.58	2377.52					
ICP-15703 x ICP-15703	Selfing	1782.35	2579.86	2593	2610.5	2625.66	2438.27					
ICP-15663 x ICP-15663	Selfing	1834.92	3152	3167.94	3183.2	3196.15	2906.84					
Grand Mean of selfed							2890.15					
	Direct Cross							Reciprocal cross				
		1HAP	8HAP	24HAP	48HAP	60HAP	Mean	1HAP	8HAP	24HAP	48HAP	60HAP
PADT-16 x PUSA-992	Intervarietal	1845.47	3415.72	3438.86	3454.14	3475.42	3125.92	1686.22	3347.52	3353.92	3367.87	3380.4
ICP-15683 x ICP-15703	Intervarietal	1335.2	2746.35	2776.58	2789.75	2805.6	2490.7	1405.69	2623.72	2638.47	2650.84	2663.7
Grand Mean of intervarietal direct crosses							2808.31					
PADT-16 x ICP-15683	Interspecific	430.54	1501.84	2681.67	3318.25	3347.66	2255.99	565.5	1973.66	2682.4	2700.38	2711.8
PADT-16 x ICP-15703	Interspecific	218.72	1080.25	2057.12	3405.86	3427.94	2037.98	361.84	1248.66	1986	2613.56	2635.3
PADT-16 x ICP-15663	Interspecific	150.34	674.77	1341.8	2532.65	2756.38	1491.19	294.88	938.92	1650.45	3287.68	3302.5
PUSA-992 x ICP-15683	Interspecific	652.69	1821.45	3363.77	3385.53	3402.65	2525.22	710.82	2750	2764.25	2779.17	2793.5
PUSA-992 x ICP-15703	Interspecific	341.38	1638.25	2773.86	3347.62	3376.48	2295.52	398.16	1861.77	2489.38	2500.45	2513.2
PUSA-992 x ICP-15663	Interspecific	275.8	1025.65	1830.75	2465.28	2688.32	1657.16	559.7	1787.36	3082.24	3105.62	3118.3
ICP-15683 x ICP-15663	Interspecific	193.24	527.51	1264.88	1717.53	1952.38	1191.11	511.16	875.66	1921.35	3220.58	3248.7
ICP-15703 x ICP-15663	Interspecific	467.6	1305.58	1747.33	2559.84	2580.38	1732.15	787.43	2156.28	3066.18	3083.62	3100
Grand Mean of interspecific crosses							1898.29					

Pollen Tube Abnormalities

It is clear from the Table 3 that in self crosses of different genotypes no pollen tube abnormality at any stage was observed. The different types of abnormalities obtained in present research are presented in Fig. 1 (c,d,e,f,g,h). In the direct and reciprocal crosses between two cultivated genotypes *i.e.* PADT-16 and Pusa-992, no pollen tube abnormality was observed. However, in the direct cross between secondary gene pool genotype *C. scarabaeoides* ICP-15683 and ICP-15703, 22.26% pollen tube abnormalities were observed at 8 HAP which decreased to 10.68% at 24 HAP and interestingly at 48 and 60 HAP no pollen tube abnormality was observed. In the reciprocal cross between ICP- 15703 x ICP-15683 at all the time intervals no pollen tube abnormality was recorded. In the direct and reciprocal interspecific crosses no pollen tube abnormality was observed at 1 HAP. Pollen tube abnormalities were recorded in most of the direct and reciprocal interspecific crosses at 24, 48 and 60 HAP. Among the direct interspecific crosses maximum pollen tube abnormalities were observed at 48 HAP in cross of *C. scarabaeoides* ICP-15683 x *C. platycarpus* ICP-15663 (43.52%) followed by the cross PADT-16 x *C. platycarpus* ICP-15663 (43.15%) at 60 HAP. Among the direct interspecific crosses it was further noticed that when cultivated indeterminate genotype Pusa-992 was involved as female parent and secondary gene pool species *C. scarabaeoides* (ICP-15683 and ICP-15703) and tertiary gene pool species *C. platycarpus* ICP-15663, as male parent, the pollen tube abnormalities at 60 HAP were low as compared to the crosses when cultivated determinate genotype PADT-16 was involved as female parent and secondary and tertiary gene pool genotypes as male parent. It was also clear from the Table 3 that in the interspecific crosses involving primary and secondary gene pool species, the pollen tube abnormalities were low at 60 HAP when cultivated genotypes PADT- 16 and Pusa-992 were used as female parent and secondary gene pool species *C. scarabaeoides* ICP-15683 as male as compared to *C. scarabaeoides* ICP-15703 as male parent. In most of the direct interspecific crosses the pollen tube abnormalities were maximum at 48 HAP, however, in majority of them, pollen abnormalities reduced considerably at

60 HAP. It is also evident from the Table 3 that pollen tube abnormalities were more 60 HAP in the direct crosses involving cultivated genotypes (PADT-16 and Pusa-992) and tertiary gene pool species *C. platycarpus* ICP-15663 as compared to the crosses involving cultivated genotypes and secondary gene pool species *C. scarabaeoides* ICP-15683 and ICP-15703. Among reciprocal interspecific crosses highest pollen tube abnormalities were recorded at 48 HAP in most cases, however, in some cases pollen tube abnormalities were highest at 24 HAP. Interestingly, in all the reciprocal interspecific crosses pollen tube abnormalities lessened with increased time interval at 60 HAP. Among the reciprocal interspecific crosses highest pollen tube abnormalities were observed in the cross *C. scarabaeoides* ICP-15703 x PADT- 16 (30.27%) followed by cross *C. platycarpus* ICP-15663 x PADT-16 (28.74%) at 60 HAP. However, in the cross *C. scarabaeoides* ICP-15683 x Pusa-992 and *C. platycarpus* ICP-15663 x *C. scarabaeoides* ICP-15703 all the pollen tube abnormalities observed at 8, 24 or 48 HAP disappeared at 60 HAP.

Table 3
The pollen tube abnormalities among self, intervarietal and interspecific crosses of pigeonpea

Self/cross combinations	Nature of cross	Pollen abnormality											
		1HAP		8HAP		24HAP		48HAP		60HAP		Mean	
PADT-16 x PADT16	Selfing	0		0		0		0		0		0	
PUSA-992 x PUSA992	Selfing	0		0		0		0		0		0	
ICP-15683 x ICP-15683	Selfing	0		0		0		0		0		0	
ICP-15703 x ICP-15703	Selfing	0		0		0		0		0		0	
ICP-15663 x ICP-15663	Selfing	0		0		0		0		0		0	
Grand Mean of selfed												0	
		Direct crosses						Reciprocal crosses					
		1HAP	8HAP	24HAP	48HAP	60HAP	Mean	1HAP	8HAP	24HAP	48HAP	60HAP	Mean
PADT-16 x PUSA-992	Intervarietal	0	0	0	0	0	0	0	0	0	0	0	0
ICP-15683 x ICP-15703	Intervarietal	0	22.26	10.68	0	0	6.58	0	0	0	0	0	0
Grand Mean of intervarietal direct crosses							3.29						0
PADT-16 x ICP-15683	Interspecific	0	11.32	17.64	26.38	15.72	14.21	0	25.52	34.35	18.4	10.66	17.78
PADT-16 x ICP-15703	Interspecific	0	0	13.49	38.72	24.54	15.35	0	19.5	25.35	39.4	30.27	22.9
PADT-16 x ICP-15663	Interspecific	0	0	20.85	37.48	43.15	20.29	0	25.88	32.66	42.85	28.74	26.02
PUSA-992 x ICP-15683	Interspecific	0	0	26.66	14.35	0	8.2	0	13.75	45.28	22.85	0	16.37
PUSA-992 x ICP-15703	Interspecific	0	0	35.82	21.42	19.75	15.39	0	0	0	28.15	13.44	8.31
PUSA-992 x ICP-15663	Interspecific	0	18.46	26.5	31.28	36.53	22.55	0	0	18.38	27.29	20.45	13.22
ICP-15683 x ICP-15663	Interspecific	0	0	20.44	43.52	33.48	19.48	0	0	30.75	35.18	9.84	15.75
ICP-15703 x ICP-15663	Interspecific	0	0	14.72	30.69	18.51	12.78	0	22.35	28.66	15.57	0	13.31
Grand Mean of interspecific crosses							16.03125						16.7075

Molecular diversity analysis among cultivated and wild genotypes of pigeonpea

In the present study an attempt had been made to find the genetic relationship between cultivated *C. cajan* (PADT-16 and PUSA-992), and wild accessions of *C. scarabaeoides* (ICP-15683 and ICP-15703) and *C. platycarpus* (ICP-15663) by using 30 SSR markers. Out of thirty SSR marker used, sixteen markers were found to be polymorphic between the genotypes under study. These markers yielded a total of thirty five alleles out of which thirty four were found to be polymorphic between the genotypes under study (Table 4). Marker ASSR 390 (PIC = 0.96) was found as most informative primer followed by ASSR 1 (PIC = 0.92), ASSR 66 (PIC = 0.90), ASSR 363 (PIC = 0.90), ASSR 379 (PIC = 0.90) and CCB4 (PIC = 0.90). In the present study SSR marker CCB10 showed least PIC

value of 0.48. Binary data was subjected to analysis by using NTSYS 2.01 software for generation of similarity matrix by SIMQUAL option. Unweighted pair method cluster analysis was carried out using Jaccard's similarity coefficient matrices determined from SSR markers to produce a dendrogram for genotypes. The coefficient of similarity ranged from 0.26 to 0.83 suggested a relatively large genetic diversity among the genotypes (Fig. 2). The markers clearly divided the used genotypes in two group at Jaccard's similarity coefficient of 0.26, first major group A containing cultivated genotypes *i.e.* PUSA-992 and PADT-16 while the second major group B contains the wild *C. scarabeoides* ICP-15683 and ICP-15703 and *C. platycarpus* ICP-15663. At 0.77 similarity coefficient the major group A was further divided at into two sub group A_1 (PUSA-992) and A_2 (PADT-16) and at 0.70 the major group B was divided into two sub groups B_1 (ICP-15683 and ICP-15703) and B_2 (ICP-15663).Sub group B_1 was further divided into B_{11} (ICP-15683) and B_{12} (ICP-15703) at 0.83. In the present study, wild and cultivated species clearly grouped under different clusters as expected, since they were well differentiated morphologically and genetically and among the wild species *C. scarabaeoides* showed more close relationship with cultivated *C. cajan* compared to wild *C. platycarpus*. Maximum diversity was found between cultivated (PADT-16 and PUSA-992) and wild *C. Platycarpus* ICP-15683 (0.13) and closest relationship was found between *C. scarabaeoides* accessions ICP-15683 and ICP-15703 (0.83). The present results indicated that sufficient genetic variation existed in the experimental material and molecular markers were effective to find out the genetic diversity between wild and cultivated types.

Table 4
Polymorphism features of SSR markers exhibiting polymorphism in the present study (range of allele sizes, number of alleles and PIC values)

Marker	AlleleSize Range(bp)	Number of alleles amplified	Number of polymorphic alleles	PIC Value
ASSR 1	100–120	3	3	0.92
ASSR 3	100–110	2	2	0.74
ASSR 23	120–200	3	3	0.85
ASSR 66	130–140	2	2	0.90
ASSR 93	100–110	2	2	0.74
ASSR 229	100–150	2	2	0.84
ASSR 363	190–200	2	2	0.90
ASSR 379	120–140	2	2	0.90
ASSR 390	190–200	2	2	0.96
ASSR 281	120–140	2	2	0.84
ASSR 148	100–110	2	2	0.84
ASSR 352	100–120	2	2	0.80
CCB4	220–600	2	2	0.90
CCac003	150–180	2	2	0.66
CCB10	100–190	2	1	0.48
CCcta001	120–650	3	3	0.84
Total		35	34	

Discussion

The study on pollen germination suggested that in self-crosses, direct intervarietal and interspecific and in respective reciprocal crosses, pollen germination increased progressively from 1 hour to 60 hours. Pollen germination percentage in self-crosses was found to be highest among all cross combinations. High pollen germination was recorded in selfed crosses because of pistil recognition system at stigmatic surface due to which compatible pollen germinates better in response to a sugary fluid secreted by mature stigma. Lipids at stigmatic surface may also stimulate pollen germination for compatible pollen (Wang et al. 2010 and Mizuta and Higashiyama, 2018). Similar to the findings of present study, the high pollen germination was recorded in selfs as compared to interspecific crosses earlier in *Cajanus* by Singh, 1996; in *Eucalyptus* by Ellis et al. 1991 and in *Gossypium* by Ram et al. 2007. Pollen germination was found to be more in intervarietal crosses of primary gene pool as compared to intervarietal crosses of secondary gene pool due to the presence of less crossability barriers among the species of primary gene pool. These barriers creates disharmony between pollen - pistil interactions and as a result pollen may not be able to adhere on the surface of stigma, even if it adhere, it may not be able to hydrate and ultimately desiccates. It is important to note that among intervarietal crosses involving determinate (PADT-16) and indeterminate (PUSA-992) type, it was better to use determinate type as female as it resulted in more pollen germination. The comparison of interspecific primary x secondary, primary x tertiary and secondary x tertiary gene pool revealed that, in general, mean pollen germination was higher in primary x secondary crosses followed by secondary x tertiary crosses and primary x tertiary crosses in both direct and reciprocal crosses. This may be due to large extent of genetic diversity between the species of primary and tertiary gene pool as compared to primary and secondary gene pool. The earlier studies on karyotype asymmetry index and seed protein analysis suggested that *C. scarabaeoides* possess more genomic similarities with cultivated *C. cajan* as compared to *C. platycarpus* (Reddy, 1981). The pachytene chromosome analysis of hybrids generated by crossing *C. cajan* with *C.*

scarabaeoides further revealed that out of eleven bivalents, eight were homologues which again indicated the similarities among these two species resulting in higher pollen germination as compared to *C. platycarpus* (Pundir and Singh, 1984). The use of molecular markers also suggested that large genetic differences occur between *C. cajan* and *C. platycarpus* (Sahu et al. 2016). Very low pollen germination in crosses involving Pusa 992 and PADT-16 with ICP-15663 suggested that these species have distant lineage from their original ancestor and are reproductively isolated from each other. These species exhibited predominance of fertilization barriers as they might possess different gene combinations of the parental species. Mallikarjuna and Moss (1995) reported low pollen-stigma compatibility in *C. cajan* and *C. platycarpus* and suggested that hybrids between these two can be obtained by embryo rescue under *in vitro* conditions. The present findings indicated that introgression of genes from *C. platycarpus* to *C. cajan* was not successful as pollen germination percent was very low between them and therefore *C. platycarpus* x *C. scarabaeoides* crosses could be used as bridge cross to transfer desirable characters from *C. platycarpus* to *C. cajan*. Percentage of pollen germination in *C. platycarpus* x *C. scarabaeoides* was comparatively higher than *C. platycarpus* x *C. cajan*. The *C. scarabaeoides* pollens show better compatibility on the stigmatic suface of *C. cajan* as compared to *C. platycarpus*. The intercross compatibility between cultivated *C. cajan* and wild *C. scarabaeoides* were also earlier observed by Yadav and Padmaja (2002). Similar incompatibility was also reported earlier by Mallikarjuna and Moss (1995) and Singh (2003) in *Cajanus* and Bharath (2018) in *Cicer*. Since *C. scarabaeoides* and *C. platycarpus* are wild types, the crossability barrier may not be very strong between them. The low croassability between *C. cajan* and *C. platycarpus* as compared to *C. cajan* and *C. scarabaeoides* in interspecific crosses was also reported earlier by Pundir and Singh (1985), Jayaprakash (1998), Singh and Bajpai (2004), Thiruvengadam and Muthiah (2007).There was good pollen germination if female parent was chosen from higher gene pool (secondary or tertiary) and primary gene pool was used as pollen parent.

In case of pollen tube length, in the selfs, intervarietal and interspecific crosses pollen tube length increased progressively from 1 hour to 60 hours in both direct and reciprocal crosses. The cultivated genotypes possess long pollen tube as compared to wild types. The reason for long pollen tube in case of *C. cajan* as compared to *C. scarabaeoides* and *C. platycarpus* is the difference in style length as flowers of wild species of pigeonpea are smaller than cultivated *C. cajan*. The pollen tube growth rate was high when cultivated determinate variety PADT-16 was used as female parent in intervarietal crosses. In case of interspecific crosses between cultivated genotypes of primary gene pool and wild genotypes from tertiary gene, pool pollen tube failed to reach the ovule and high level of incompatibility was observed. The high level of incompatibility may be due to non-harmonious pollen tube and pistil interaction. The results of interspecific crosses, indicated that pollen tube length was more when cultivated type is used as female and wild type as male. The probable reason may be the difference in style length of flowers of wild and cultivated type, as flowers of wild type are smaller than cultivated type.

In case of pollen tube abnormality, no abnormality was observed in hand-selfing in all species. The absence of pollen abnormalities in selfed crosses may be due to harmonious interaction between pollen and pistil. Maximum mean pollen abnormality was observed in cross ICP-15663 x PADT-16. In cross between cultivated *C. cajan* and wild *C. scarabaeoides*, low level of barriers was recorded when *C. scarabaeoides* was used as female in crossing. Low level reproductive barriers were recorded in cross PADT-16 x ICP-15683 (*C. scarabaeoides*) compared to PADT-16 x ICP-15703 (*C. scarabaeoides*). The abnormalities *viz*. bursting of pollen tubes, coiling of tubes, penetration of tubes in lateral style region was observed. The abnormalities were not specific to cross and time interval. The pre-fertilization barriers were not very strong in cross between *C. cajan* and *C. scarabeoides*, as the pollen tubes reached to ovary in both direct as well as reciprocal crosses. However, in cross between *C. cajan* and *C. platycarpus*, pollen tube failed to reach to ovule reflecting presence of strong pre-fertilization barriers but in its reciprocal cross (*C. platycarpus* x *C. cajan*) pollen tube successfully reached to ovule (at 48 HAP) showing low intensity of crossability barriers. In interspecific crosses involving *C. platycarpus* (ICP-15663) as female parent, high pollen germination was observed in *C. platycapus* x *C. scarabaeoides* compared to *C. platycapus* x *C. cajan* and among all of them highest pollen germination was observed in cross ICP-15663 x ICP-15703. Hogenboom (1972) recorded abnormalities in intraspecific crossings, most probably due to inhibitory action of incompatible genes, which contributes to non-functional partner relationship, although successful fertilization is possible. It was observed that when cultivated indeterminate variety Pusa 992 was used as female in interspecific crosses, the pollen tube abnormalities were low. This may be due to the fact that nature prefers the indeterminate types. Since secondary and tertiary gene pool genotypes are indeterminate and cultivated Pusa 992 as female, is also indeterminate and hence, in the interspecific crosses involving these, the pollen tube abnormalities were low as they may have better cross compatibility. Present findings were similar to the earlier findings of Pundir and Singh (1985) who also observed that pollen tubes growth were inhibited at stigma and in style of cultivated *C. cajan* in interspecific cross with *Atylosia* spp and observed abnormalities like bulging of tips of pollen tubes, club shaped pollen tube tips. Singh and Bajpai (2004) also observed abnormalities in cross *C. cajan* x *C. platycarpus* and *C. cajan* x *C. volubilis*. The abnormalities *viz*. intercoiling of pollen tubes, bursting of pollen tubes, growth of pollen tubes away from stigmatic surface, growth of tube from two or more places from single pollen, pollen tube penetrating the style from lateral region may be due to presence of fertilization barrier in stigma and upper style portion. Present findings are in agreement with the earlier findings of Jayaprakash (1998), Singh and Bajpai (2004) in *Cajanus* and Ahmad et al. (1988) in *Cicer*. Hodnett et al. (2005) indicated that adverse pistil– pollen interactions that include the tube growth inhibition in wide crosses may be viewed as a consequence of inharmonious genetic interactions due to genetic divergence of the species involved.

In the present study use of molecular markers clearly differentiated wild species (*C. scarabaeoides* and *C. platycarpus*) from cultivated (*C. cajan*) species under different clusters. Among wild species *C. scarabaeoides* showed more close relationship with cultivated *C. cajan* compared to wild *C. platycarpus*. Present findings of grouping cultivated and wild types into different clusters were supported by the previously recorded taxonomic Van der Maesen (1980) and cytological Pundir and Singh (1985) findings. The present results indicated that sufficient genetic variation existed in the experimental material and molecular markers are an effective tool to find out the genetic diversity between wild and cultivated genotypes. High molecular diversity between cultivated and wild accessions of pigeonpea have been reported earlier by Songak et al.(2010), Mudaraddi et al.(2013), Magdum (2014), Oinam et al.(2015) and Sharma et al. (2020) using SSR markers.

Conclusions

In order to introgress desirable genes from wild species into cultivated pigeonpea, pre breeding efforts are required. These pre breeding efforts are severely hampered by different fertilization barriers. In the present study interspecific crosses showed low pollen germination as well as pollen tube growth as compared to both selfed and intervarietal crosses. In general, mean pollen germination was higher in primary x secondary crosses followed by secondary x tertiary crosses and primary x tertiary crosses in both direct and reciprocal crosses. In case of interspecific crosses pollen tube length was more when cultivated type is used as female and wild type as male. More pollen tube abnormalities were observed in interspecific crosses involving cultivated and species from tertiary gene pool. The estimation of molecular diversity through SSR markers indicated that maximum diversity is present between cultivated *C. cajan* and wild *C. platycarpus* whereas closest relationship was observed between *C. scarabeoides* ICP-15683 and ICP-15703.

Declarations

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Competing Interest

The authors have no relevant financial and non financial interest to disclose.

Authors Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Anandi Karn, Sanjay Kumar Verma, Ravindra Kumar, Usha Pant, Anju Arora, Ram Kumar Sharma and Amit Kumar Gaur. The first draft of the manuscript was written by Anandi Karn, Sanjay Kumar Verma, Ravindra Kumar, Usha Pant, Anju Arora, Ram Kumar Sharma and Indra Prakash Singh and all authors commented on previous version of the manuscript. All authors read and approved the final manuscript.

Data availability

The data sets generated and/ or analysed during current study are available from the corresponding author on reasonable request.

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Supplementary Files

Supplementary Table 1 is not available with this version.

Figures

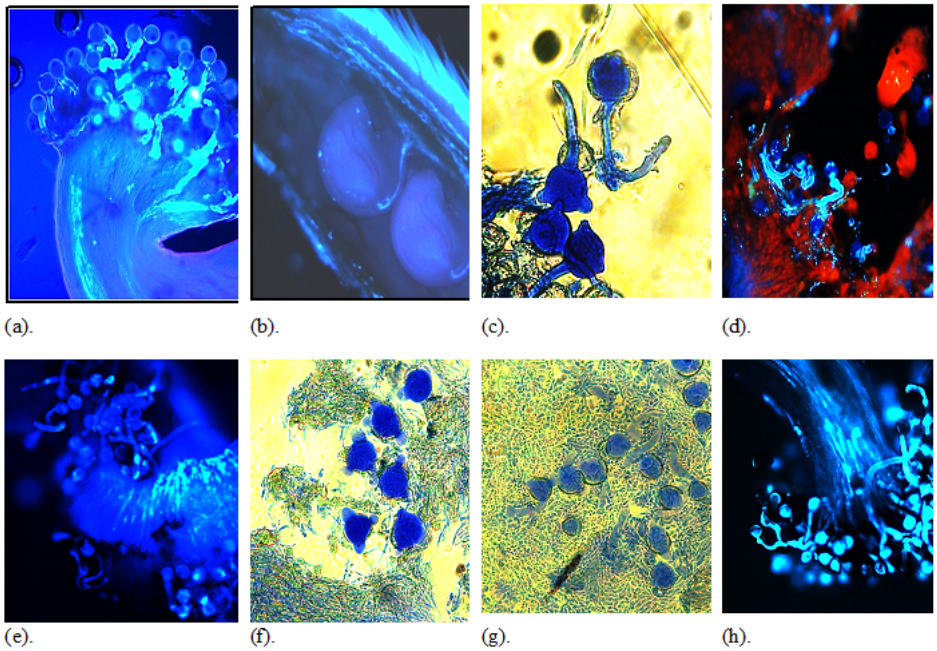

Figure 1

The microscopic images showing (a) . Germination of pollen on stigma in self of Pusa 992, (b). Pollen tubes reaching into ovules in self cross of PADT-16, (c). Abnormal J shaped pollen tube in interspecific cross ICP-15703 x PADT-16, (d). Abnormal Zig-zag shape pollen tubes of interspecific cross ICP-15703 x Pusa-992, (e). Inter coiling of pollen tubes in interspecific cross PUSA-992 x ICP-15683, (f). Two or more pollen tube growth from single pollen grain in interspecific cross PADT-16 x ICP-15663, (g). Coiling and bending back of pollen tube in ICP-15683 x ICP-15703 cross, (h). Pollen tube growth away from stigmatic surface in interspecific cross ICP-15663 x ICP-15703.

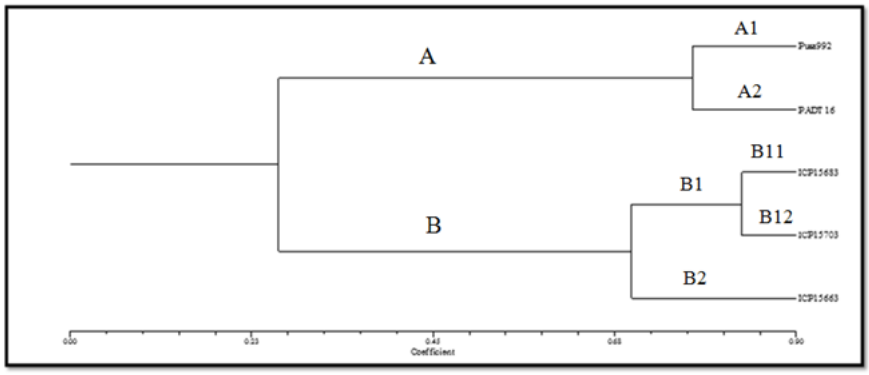

Figure 2

Dendrogram showing genetic relationship between wild and cultivated genotypes of pigeonpea