Electronic Journal of Plant Breeding, 8(1): 63-71 (March 2017)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2017.00009.6

Research Article

Identification of inter-specific hybrid between *Cajanus cajan* (L.) and *C. cajanifolius* (H.) using cyto-morphological and DNA markers

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(Received: 12 May 2016; Revised: 17 March 2017; Accepted: 20 March 2017)

Abstract
Cajanus cajanifolius, the putative progenitor of domesticated *C. cajan*, has many agro-economic traits those need to be introgressed into the cultivars of pigeonpea to widen its genetic base. In this study interspecific F_1 hybrids, involving *C. cajan* (ICPL 87119) and *C. cajanifolius* (ICPW 31), were raised, and their response to bruchid infestation also assessed. Subsequently, the hybrids were characterized using the morphological, cytological and DNA markers based attributes. The F_1 hybrids showed resistance to bruchid infestation caused by *Callosbrochus maculatus* and *C. chinensis*. The morphological traits of F_1 hybrids with respect to their parents revealed that these were intermediate between the parents with predominance of the characteristics of both the parents. The pollen mother cells (PMC) obtained from the F_1 hybrids showed regular meiosis involving 11 bivalents in majority of the cases which affirmed the genetic homology between these two species. But, heteromorphism was noticed for two bivalents during diakinesis. DNA marker (SCoT and SSR) analysis also revealed polymorphic fragments between the parents and their inheritance to the putative F_1 hybrid. In conclusion, the simultaneous use of cyto-morphological analysis and DNA marker based genotyping demonstrated the genetic divergence between the parental genotypes with contrasting response to bruchid infestation and the hybridity of the F_1 plants with accuracy.

Key words
DNA marker, bruchid resistance, hybrid identification, pigeonpea

Introduction

Cajanus cajan (pigeonpea) is a protein rich pulse crop widely cultivated in the tropics and semi-arid tropics of the world. India has 5.06 million hectares land under pigeonpea farming. India alone contributes about 3.29 million tonnes i.e. 67.7 % of the world production, with a yield of 650 Kg/ha (FAOSTAT, 2016; faostat3.fao.org/compare/E). However, the productivity and yield of pigeonpea is constantly being affected by the biotic stresses laid upon by the field pests and diseases *vis-a-vis* the storage pests. As a consequence, there is a huge shortage of pigeonpea grains in India during last couple of years which to led escalation of price for these grains as well as it became unaffordable to a large sector of consumers for whom pigeonpea grains are core source of protein supplements. The storage pests, bruchids (*Callosbrochus spp*.) also adversely affect the yield, economy of the pigeonpea grains, and reduce the nutritional value in both storage conditions and to some extent in the field conditions. The allele(s) conferring host resistance against these pests are not available in the cultivated genotypes. But, *C. cajanifolius* genotypes, the putative progenitor species of domesticated pigeonpea (Mallikarjuna *et al.*, 2012), possessed the genes for various agro-economic traits including bruchid resistance, high protein content and moderate drought tolerance (Panigrahi *et al.*, 2001). These agro-economic trait(s) including bruchid resistance could be introgressed into the cultivated *C. cajan* background through inter-specific hybridization aiming at development of cultivars conferring bruchid resistance in pigeonpea.

The success of introgressive hybridization relied upon the F_1 hybrids, because these hybrids are starting material for the production of advanced breeding lines and expansion of genetic base of a crop, in particular the monotypic crop like pigeonpea. Already some attempts were made to generate interspecific hybrid between *C. cajan* and *C. cajanifolius* (Mallikarjuna *et al.*, 2012 and Panigrahi *et al.*, 2001) and success was also achieved. Identification and characterization of true hybrids is mandatory in inter-specific crosses, and is more desiderated for often cross pollinated crop like pigeonpea. Cyto-morphological analysis to ascertain hybridity is a common practice in various crops including pigeonpea. However, these cyto-morphological attributes have limited reproducibility due to environmental influences and developmental variations which limit their applicability in introgressive hybridization programmes. As a consequence, DNA markers were introduced in conjunction to cyto-morphological attributes for the identification of hybrids with precision and characterization at the early stages of development. In the last couple of decades, a large number of DNA markers including random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphism (SNP) markers were

DOI: 10.5958/0975-928X.2017.00009.6

developed in pigeonpea. But only few of them have been employed for hybrid identification purpose in *Cajanus* species. Reports were also available on characterization of *C. cajan* × *C. cajanifolius* hybrids using cyto-morphological attributes (Mallikarjuna *et al.,* 2012) and protein profiling (Panigrahi *et al.,* 2001).

In the present study, we developed an interspecific F_1 hybrid, involving two genotypes with contrasting host response to bruchid *viz. C. cajan* (ICPL 87119) and *C. cajanifolius* (ICPW 31), and ascertained the hybridity by analysing cyto-morphological attributes and inheritance of parent specific DNA (SCoT and SSR) markers.

Materials and methods

Plant materials: A total of 34 genotypes of 12 species of the genus *Cajanus* were obtained from ICRISAT, Patancheru, Hyderabad, and maintained at the experimental garden, School of Life Sciences, Sambalpur University, Odisha, India. All the genotypes were subjected to screening for bruchid resistance as per the protocol developed by Amusa *et al.* (2013). Based on their response to bruchid infestation, *C. cajanifolius* (ICPW 31) from the secondary gene pool and *C. cajan* (ICPL 87119) from the primary gene pool were chosen as donor and recipient parent, and the inter-specific F_1 hybrids were raised.

Morphological characterization: Morphological parameters such as time taken for seed germination, growth habit, branching pattern, leaf morphology, plant height, number of primary branches, basal petal colour, pattern of streaks on the petal, days to first flowering, pod colour, pod size, pod constriction, locules per pod, seed coat colour, seed shape, presence/absence of seed aril and 100-seed weight were studied on the F_1-hybrids along with their parents. The parent specific morphological attributes were used as markers to characterize the F_1 hybrids.

Cytological studies: Meiotic analysis of pollen mother cells (PMCs) of the F_1 hybrids along with their parents was performed to study the chromosome homology. Anthers containing PMCs were fixed in ethanol: acetic acid (3:1) and were squashed in 2% aceto-carmine. Well spread preparations of PMCs were used for the observation of different stages like diakinesis, metaphase-I and anaphase-I under microscope. A total of 84 PMCs were analysed from each parents, and F_1 hybrids.

DNA marker analysis: Genomic DNA from both the parents [*C. cajanifolius* (ICPW 031) and *C. cajan* (ICPL 87119)] and one of the F_1 hybrids were isolated and purified using the standard protocol developed by Sivaramakrishnan *et al.* (1997) with few modifications.. Quantity and quality of the isolated DNA samples were assessed and equilibrated to 10 ng/µl using UV-spectrophotometer (UV1, Thermo, UK), and validated through agarose gel based visualisation using uncut phase lambda DNA as standard.

The hybridity of the F_1 plants were assessed by using twenty SCoT (Start Cordon Targeted Polymorphism, Table 2; Collard and Mackill, 2009) and ten pair of SSR (Simple Sequence Repeats, Table 3; Odeny *et al.*, 2007) primers. For SCoT marker analysis, PCR amplification was performed in a volume of 23 µl reaction mixture containing 20 ng of template DNA, 0.25 µM of primer, 2.5 µL 10X assay buffer, 1 µL dNTPs (2.5 mM each), 0.5 µL $MgCl_2$ (20 mmol/L), 0.33 µL Taq (1.67U) DNA polymerase and 14.67 µL of ddH_2O. PCR reactions were initiated with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After 35 cycles of PCR reaction, a final extension step at 72°C for 5 min was performed. For SSR marker analysis, PCR amplification was carried out in 10 µL of reaction mixture containing 5 ng template DNA, 4.2 µL sterile milliQ H_2O, 1 µL of 10X assay buffer, 1.6 µL dNTPs mix (2.5mM each), 1 µL $MgCl_2$ (25mM), 0.33 µL Taq polymerase (1.0 U) and 0.3 µL (10pmol) of each forward and reverse primers. PCR reactions were started by initial denaturation of template DNA at 95°C for 5 min followed by a touchdown PCR protocol of 10 cycles (denaturation at 94°C for 20 sec, annealing at 57°C for 20 sec and extension at 72 °C for 30 sec) involving a reduction of 1°C in each cycle. Further, it was followed by 30 cycles of denaturation, annealing and extension each at 94°C for 20 sec, 48°C for 20 sec and 72°C for 30 sec, respectively. Final extension was at 72°C for 20 sec.

Amplified PCR products of both the SCoT and SSR primers were separated by electrophoresis in 1.4% and 4% agarose gels, respectively; visualised under the gel documentation system (Fire Reader, UVITEC, Cambridge) and photographed. The sizes of the amplified fragments were determined using 250 bp and 100 bp step up ladder (Bangalore Genei Pvt. Ltd.) respectively, and Total Lab-120 software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, UK). The parental polymorphism (%) detected from each SCoT primers and SSR primer pairs were calculated.

Results and discussion

Ascertaining the hybridity of the putative F_1 plants at an early stage is a prerequisite in the studies pertaining to wide introgressive hybridization as it acts as starting material for all subsequent breeding endeavours (Mishra *et al.,* 2012). In this study, the F_1 hybrids raised from the cross *C. cajan* × *C. cajanifolius* were characterized using the

DOI: 10.5958/0975-928X.2017.00009.6

morphological, cytological, and DNA marker analysis.

Morphological characterization: The morphological attributes of F_1 hybrids was conglomeration of the contrasting traits with respect to their parents. The *C. cajanifolius* (ICPW-31) and F_1 had the presence of dense small trichomes on their leaves making them velvety to touch, and were comparable to that of cultivated pigeonpea (ICPL 87119) which had trichomes on their leaves but they were not as much velvety to touch. Variation was observed with respect to the petal colour and streaks on the petals (Table 1). The basal petal of *C. cajanifolius* was less yellowish than that of the *C. cajan* and F_1 (Fig 1c). Both *C. cajan* and the F_1 hybrids were found erect with spreading type of branching pattern while in *C. cajanifolius* semi-spreading branching pattern was observed (Table 1). Regarding plant height, *C. cajan* was taller than both the F_1 and *C. cajanifolius*. The number of primary branches varied comparatively from 9 to 10 in *C. cajan*, 4 to 5 in *C. cajanifolius* and 6 to 7 in the F_1 hybrids. Pod morphology was also diverged among the *C. cajan*, F_1 and *C. cajanifolius*. Pod constriction was prominent on *C. cajan*, intermediate on F_1, and slightly constricted on *C. cajanifolius*. The locules between the consecutive seeds were more prominent in *C. cajanifolius* with clear cut demarcations but were least prominent in both the *C. cajan* and the hybrids. Pod length too varied from 3.4 to 4 cm in *C. cajanifolius*, compared to a pod length of 5.4 to 5.9 cm in *C. cajan* and 3.73 to 4.11cm in the F_1 hybrids.

Major distinction between *C. cajanifolius* and *C. cajan* was the seed coat colour (Fig 1e) and seed aril. Seed coats of *C. cajan* were orange in colour while seed coats of both the F_1 hybrids and *C. cajanifolius* were found mosaic ash brown in colour. Seed aril was prominent in both the *C. cajanifolius* and F_1 while it was absent in *C. cajan*. These findings supported the earlier observation made by Malikarjuna *et al.* (2012) except the results obtained on plant height and number of primary branches. The exceptions might be due to the variation in accession types selected for breeding and variation in environmental conditions of the study area. Compared with their parents, the hybrids had the lowest values of pod setting which is supported by the earlier results obtained by Malikarjuna *et al.* (2012), though Pundir and Singh (1985) did not observe seed set in the hybrid of *C. cajan* and *C. cajanifolius*. With respect to most of the quantitative traits such as leaf size, pod size and 100 seed weight the hybrids showed mid parental values as observed in the hybrid of *C. cajan* × *C. cajanifolious* (Mohanty and Patnaik, 1989), and *C. cajan* × *C. scarabaeoides* (Mishra *et al.*, 2012); and highest values of time taken for germination and days to first flowering (Table 1). Most of the characters of *C. cajanifolius*, such as dense trichomes on leaves, ash brown seed coat colour, oval seed shape, prominent seed strophiole (Table 1, Figure 1a-e) were noticed in the F_1 hybrids. Similarly, features of *C. cajan* such as spreading branching pattern, about 4 to 5 locules per pod (Table 1) and deep yellow colour of basal petals were also seen in the F_1 hybrids (Fig 1c). This revealed that the F_1 hybrids were intermediate between the parents with prevalence of the characteristics of both *C. cajan* and *C. cajanifolius*.

Cytological analysis of the hybrids: Meiocytes of both the parents revealed 11 bivalents at diakinesis and metaphase-I, as the somatic chromosome number is 2n=2x=22 (Fig 2a). Meiocytes of the F_1 hybrid also showed formation of 11 bivalents at diakinesis and 11-11 separation at anaphase-I in majority of PMCs, which was an indicative of inter-genomic homeology between the parents. But in few cases (4.76%) two heteromorphic bivalents were observed which were loosely paired at diakinesis (Fig. 2b) and laggard formation at anaphase-I. Similar kind of observations were also made in *C. cajan* × *C. cajanifolius* and *C. cajan* × *C. scarabaeoides* hybrids (Mishra *et al.*, 2012, Mallikarjuna *et al.*, 2012), which are indicative of partial non-homology between certain homologues or the development of desynaptic genes in the course of genetic divergence during evolution (Mishra *et al.*, 2012). However, the number of heteromorphic bivalents observed in F_1 hybrids contradicts with earlier reports, and this might be due to different genotypes used in these studies.

DNA marker analysis: DNA marker based assessment of F_1 hybrids has distinct advantages over cyto-morphological analyses. The cyto-morphological markers, influenced by the environmental conditions, are labour intensive and more time consuming (Kumar *et al.*, 2012). Conversely, the DNA markers are least influenced by the environmental effect and revealed ample polymorphism to discriminate the closely related inbred lines. Thus DNA markers have been applied along with cyto-morphological analyses in many crop plants for the detection of parental polymorphism, genetic diversity studies, screening of hybrids and several breeding endeavours (Kumar *et al.*, 2012 and Mishra *et al.*, 2012). Among various DNA markers, SSR markers have been considered as the current markers of choice, and are mostly used for plant breeding applications, genetic diversity studies and screening of hybrids due to their high level of reproducibility, co-dominant nature, wide genome coverage and relative abundance.. Among the dominant marker systems available, SCoT based detection of polymorphism relies on differential enzymatic amplification of functional DNA fragments either in the gene or in the close vicinity of genes using PCR (Collard and Mackill, 2009).

DOI: 10.5958/0975-928X.2017.00009.6

This marker also showed moderate polymorphism and has been used for genetic mapping (Sahu *et al.*, 2015) and diversity studies (Satya *et al.*, 2015) in various crop plants.

In the present study a combination of both SCoT and SSR primers were successfully utilized for the detection of parental polymorphism and identification of the F_1 hybrids. All the twenty SCoT primers assayed revealed parental polymorphism where as nine of the ten SSR primers showed polymorphism between the parents (Table 2, 3). The occurrence of this kind of polymorphism (Fig 3) might be due to the genetic divergence between the parents at the species level. Out of the 76 polymorphic fragments generated by the SCoT primers, 54 numbers of fragments, including 29 *C. cajan* specific and 25 *C. cajanifolius* specific, were inherited to the F_1 hybrid (Table 2). Similarly, 15 number of SSR fragments, including nine *C. cajan* specific and six *C. cajanifolius* specific, were inherited to the hybrid (Table 3). The inheritance of male parent (*C. cajanifolius*) specific band to the F_1 hybrids confirmed the genuineness of the artificial crossing, and heterozygotic nature of the F_1 hybrid. The finding showed the efficacy of both the SSR and SCoT markers in ascertaining hybridity in pigeonpea with precision in concurrence to cyto-morphological markers. However, some of the parental polymorphic fragments did not appear in the F_1 hybrid (Table 2, 3), and this might be attributed to DNA recombination followed by minor genomic reorganization (Huchett and Botha, 1995), and loss of primer annealing sites due to chromosomal crossing over during meiosis (Smith *et al.*, 1996).

Details of the number of polymorphic markers identified, percentage of polymorphism and inheritance of the parent specific fragments to the hybrid are depicted in table 2 and 3. Since the objective of this pursuit is to ascertain the hybrid nature of putative inter-specific F_1 at seedlings stage, the confirmation of hybridity of the raised F_1 seedlings by screening with either SSR or SCoT markers would be practical, and of breeding significance to this crop. Previous studies have reported analysis of inter-specific hybrids, involving *C. cajan* and *C. cajanifolius*, using either morphological, cytological and/or biochemical parameters (Mallikarjuna *et al.*, 2012 and Panigrahi *et al.*, 2001). The use of these cyto-morphological and biochemical attributes are limited to environmental fluctuations, less reproducibility and low level of polymorphism. Hence, the combined use of morphological, cytological and DNA marker based characterization would strengthen the precise evaluation of F_1 hybrids and could beneficially be utilized for further studies involving genetic mapping and marker assisted breeding programmes.

Acknowledgement

The authors are highly thankful to the Department of Science and Technology, New Delhi for providing INSPIRE Fellowship to the author SKM for pursuing the doctoral degree, and acknowledged the laboratory facility accorded by the Vice-Chancellor, Sambalpur University.

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DOI: 10.5958/0975-928X.2017.00009.6

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Electronic Journal of Plant Breeding, 8(1): 63-71 (March 2017)
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Table 1. Morphological characteristics of *C. cajan* (ICPL 87119), *C. cajanifolius* (ICPW-31) and their F_1 hybrids

Morphological parameters	*C. cajan* (Ovule Parent)	F_1 hybrids	*C. cajanifolius* (Pollen Parent)
Time taken for seed germination (Days)	4.6 ± 1.1	3.8 ± 0.84	6.6 ± 0.55
Growth habit	Indeterminate	Indeterminate	Indeterminate
Trichomes on leaves	Trichomes (Less velvety to touch)	Danse trichomes (velvety to touch)	Danse trichomes (velvety to touch)
Leaf colour	Green	Dark green	Dark green
Plant height (m)	2.59 ± 0.2	2.33 ± 0.09	2.46 ± 0.08
Branching pattern	Spreading	Spreading	Semi-spreading
No. of primary branches	9.4 ± 0.55	5.4 ± 0.55	4.8 ± 0.84
Days to first flowering	133.4 ± 4.50	116.8 ± 4.50	150.2 ± 2.59
Colour of basal petal	Deep yellow	Deep yellow	Light yellow
Pattern of streaks on the petal	Sparse	Less prominent	Prominent
Young pod colour	Green with uniform brown patches	Green with brown patches at the demarcations	Greenish
Pod length (cm)	5.68 ± 0.24	3.92 ± 0.19	3.66 ± 0.30
Pod constriction	prominent	intermediate	Slight
No. of locules per pod	4 ± 0.70	3.8 ± 0.84	5. 4 ± 0.89
Seed coat colour	Orange	Ash brown	Ash brown
Seed shape	Globular	Oval	Oval
Seed strophiole	Absent	Prominent	Prominent
100 Seed weight (g)	12.26 ± 0.16	6.14 ± 0.17	3.54 ± 0.04
*Reaction to *C. maculatus*	Susceptible (7)	Resistant (1)	Resistant(1)
*Reaction to *C. chinensis*	Susceptible (8)	Resistant (1)	Resistant(1)

* The host resistance to bruchid infestation was measured in 1-9 scale

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Table 2. Amplification pattern of SCoT markers showing parental polymorphism and the inheritance of polymorphic markers

Sl. No	Primer name	Amplified fragment size (base pair)	No. of fragments amplified	Parental polymorphism (%)	Species specific inherited fragment (Fragment size in base pair)	
					C. cajan specific	*C. cajanifolius* specific
1	SCoT-3	340-2560	8	50.00	1397, 2555	1207
2	SCoT-4	410-2556	7	71.43	741	481
3	SCoT-5	410-1600	6	66.67	1597	410, 750
4	SCoT-7	430-1860	8	87.50	500, 627, 750, 1250	973, 1107, 1857
5	SCoT-8	480-1670	6	33.33	-	-
6	SCoT-9	630-1770	8	62.50	783	1479
7	SCoT-11	610-2030	6	50.00	-	1458
8	SCoT-12	410-1570	5	20.00	-	554
9	SCoT-13	390-1863	9	44.44	633, 1084, 1275, 1863	-
10	SCoT-14	778-1441	4	75.00	399, 1441	937
11	SCoT-15	465-2150	8	87.50	723, 907	465, 598
12	SCoT-18	558-1800	6	33.33	-	1636, 1800
13	SCoT-19	454-1442	5	80.00	454, 1128	1000
14	SCoT-20	329-1276	8	50.00	329, 1276,	1105
15	SCoT-21	237-1083	8	75.00	539	237, 420, 1083
16	SCoT-23	500-1128	5	40.00	655	500
17	SCoT-24	562-1684	6	50.00	708	-
18	SCoT-27	698-2000	7	57.14	1481	698, 1684
19	SCoT-30	750-2036	4	75.00	1307, 2036	750
20	SCoT-36	311-1802	6	50.00	311, 599	1192
	Total		**130**	**57.94**	**29**	**25**

Table 3. Amplification pattern of SSR markers showing parental polymorphism and the inheritance of polymorphic markers

Sl. No	Primer name	Amplified fragment size (base pair)	No. of fragments amplified	Parental polymorphism (%)	Species specific inherited fragment (Fragment size in base pair)	
					C. cajan specific	*C. cajanifolius* specific
01	CCggt001	77-1479	5	20.00	-	512
02	CCtc001	158-306	3	100.00	269	-
03	CCggc001	188-520	2	50.00	187	-
04	CCttat001	221-263	2	-	-	-
05	CCtta004	150	1	100.00	-	-
06	CCtta005	212-500	5	100.00	-	212, 241, 266, 500
07	CCac001	147-266	2	50.00	147	-
08	CCtc002	106-274	3	100.00	106, 198, 274	-
09	CCgtt001	277-936	2	100.00	277	936
10	CCat004	400-1290	3	100.00	873, 1289	-
	Total		**53**	**56.00**	**14**	**11**

Electronic Journal of Plant Breeding, 8(1): 63-71 (March 2017)
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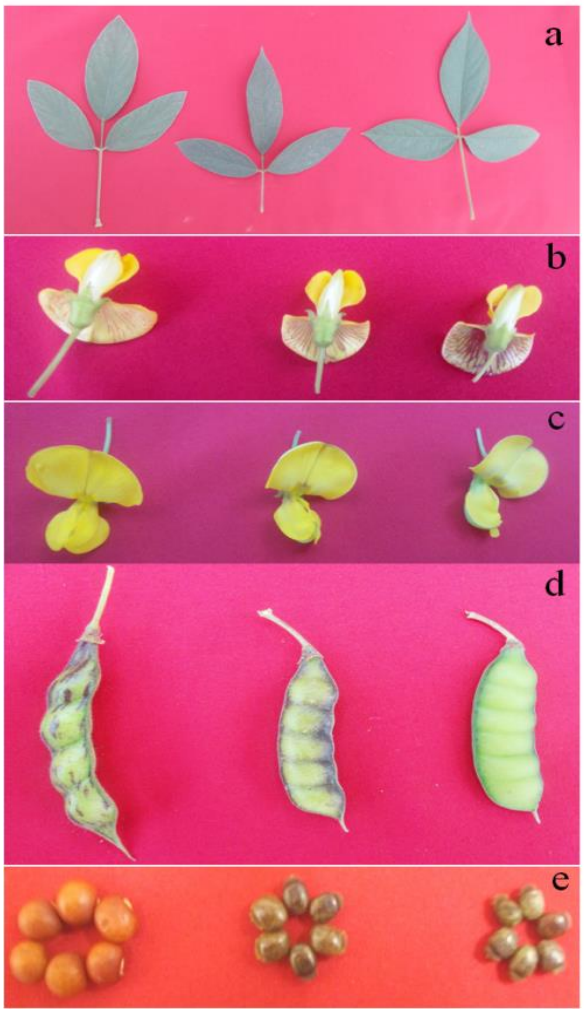

Fig. 1. Morphological characteristics of *C. cajan* (Asha), *C. cajanifolius* (ICPW-031) and F_1 hybrid
(From left to right: *C. cajan* (Asha), F_1 and *C. cajanifolius* (ICPW-031). a. Leaf morphology and trichome on leaves; b: Flower morphology and streaks on the petals; c: Flower morphology with petal colour; d: Pod morphology and pod shape; e: Seed morphology and presence/absence of strophioles)

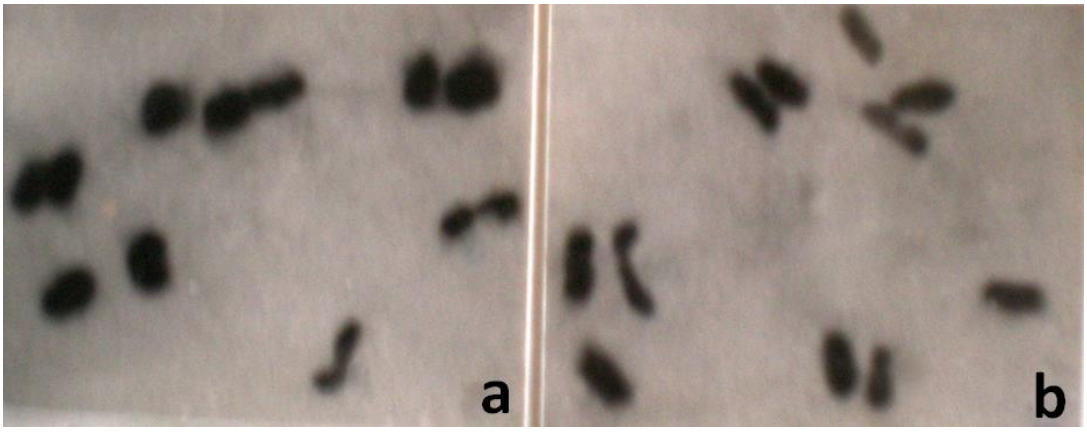

Fig. 2. Meiotic study of the F_1 hybrids between *C. cajan* (ICPL-87119) and *C. cajanifolius* (ICPW-31) depicting 11 number of bivalents

DOI: 10.5958/0975-928X.2017.00009.6

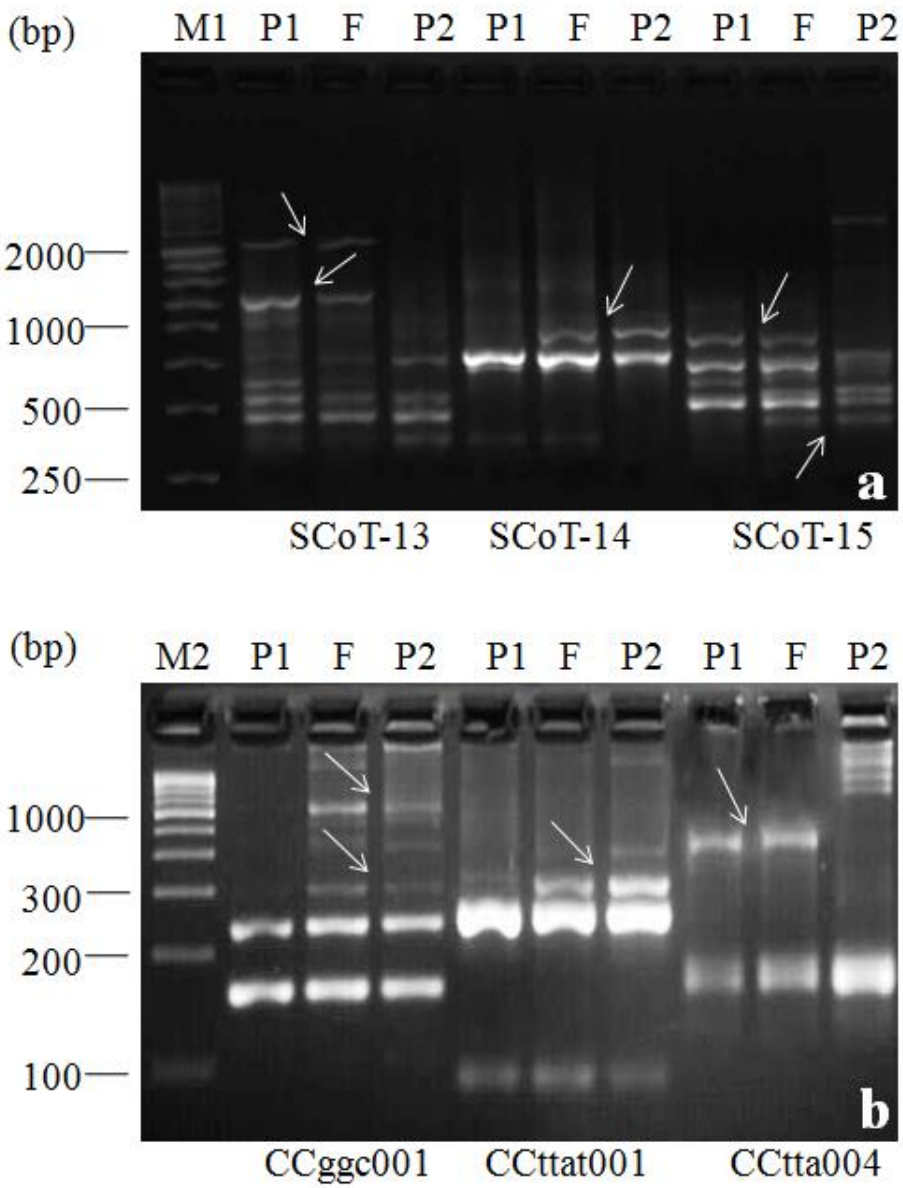

Fig. 3. Inheritance of parental polymorphic fragments, generated by three SCoT primers

(a) and three pair of SSR primers (b), to the F_1 plants (Arrow) (M1: 250 bp ladder, M2: 100 bp ladder, P1: *C. cajan*, P2: *C. cajanifolius*, F: F_1 hybrid; name of the primers also at mentioned)