Ribosomal DNA variation and phylogenetic relationships among *Cajanus cajan* (L.) Millsp. and its wild relatives

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Genomic DNA from eight species of *Cajanus* and six accessions and varieties of *Cajanus cajan* were digested with 10 restriction enzymes and probed with full length ribosomal gene (pTA71) from wheat and rDNA intergenic spacer region (IGS) flanked by 25S and 18S rDNA gene of *Vicia faba*. The length of the rDNA repeat units in *Cajanus* flanked by *Eco*RV sites was between 10.9 kb and 11.9 kb. Three rDNA repeat unit length classes were identified among the eight species. Restriction fragment length polymerphisms (RFLPs) between the species were readily detected in all the enzyme–probe combinations, however, RFLPs could not be detected between the accessions and varieties of *C. cajan*. The inter-specific RFLPs were used to construct a dendrogram for analysing the genome relationships. The dendrogram revealed a close relationship between the cultivated species *C. cajan* and the wild species *C. scarabaeoides*. Our data did not support the view that *C. cajan* could have evolved from a hybridization between *C. scarabaeoides* and *C. lineatus*. The Australian species *C. scarabaeoides* and *C. reticulatus* were closely related to the Indian species *C. cajan* and *C. platycarpus*, respectively. Therefore, the observation generalizing that Australian species of *Cajanus* are less closely related to the Indian species could not be favoured. Among the species studied, *C. goensis* and *C. lineatus* were distantly related to the cultivated species and other wild relatives.

PIGEONPEA (*Cajanus cajan* (L.) Millspaugh) is one of the major grain legumes of the tropics and subtropics. It is produced commercially by small and marginal farmers in India, Myanmar, Kenya, Malawi, Uganda and a few countries of Central America. It belongs to the family Leguminosae, subfamily Papilionideae, tribe Phaseolae and subtribe Cajaninae. After the merger of the genus *Atylosia* to *Cajanus*, the latter now has 32 species[1], of which *Cajanus cajan* is the only cultivated species. Chemical constituents in *Cajanus* were summarized[2] but it is difficult to draw taxonomic or evolutionary conclusions based on this rather inadequate information. Seed protein electrophoresis[3–5] and isoenzyme studies[6] in *Cajanus* revealed remarkable similarities between *C. cajan* and wild species indicating congenericity. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers were used to understand the relationships between *C. cajan* and its wild relatives[7,8]. The results from RFLP data supported the conclusions drawn from seed protein profiles and to a lesser extent, crossability relationships and cytology[7]. A consensus regarding the genome ralationships in *Cajanus* could not be achieved as the application of DNA markers is still at its early stages compared to other crop species.

RFLP analysis of rDNA, although not with same impact as chloroplast DNA, has proven to be of tremendous utility in phylogenetic reconstruction[9–11]. The tandem arrays of rDNA repeat units, generally located near the nucleolar organizing regions (NORs) of chromosomes, combine highly conserved regions encoding for ribosomal RNAs (18S, 5.8S, 25S), with more variable intergenic spacer (IGS) regions[12]. In addition to its value in phylogenetic reconstruction, the biparentally inherited nuclear rDNA variation also provides valuable genetic markers for the analysis of genomic relationship among cultivated species and its wild relatives[13–15]. Despite its immense potential in genome analysis, nothing is known about the structure and variation of rDNA repeat units in any species of *Cajanus*. In the present study, therefore, we have examined variations in length and restriction sites of rDNA in seven wild species, and five accessions and one cultivated variety of *Cajanus cajan* using heterologous probes from wheat and faba bean.

Table 1. Species analysed, their country of origin and the estimated rDNA repeat unit

Genotype	Country of origin	Estimated length of rDNA repeat unit (kb)
Cajanus platycarpus (Benth.) van der Maesen	India	11.5
C. scarabaeoides (L.) Thouars	Australia	10.9
C. goensis Dalz.	India	10.9
C. lineatus (W & A) van der Maesen	India	10.9
C. albicans (W & A) van der Maesen	India	11.9
C. mollis (Benth.) van der Maesen	India	11.9
C. reticulatus (Dryander) F. v. Muell.	Australia	11.9
C. cajan (L.) Millsp.	India	11.9
C. cajan ICP 6443	India	
C. cajan ICP 6974	India	
C. cajan ICP 7118	India	
C. cajan ICP 7182	India	
C. cajan ICP 7220	India	
C. cajan cv. CO 6	India	

ICP accessions were from ICRISAT, Hyderabad and the cultivated variety CO 6 was from Tamil Nadu Agricultural University, Coimbatore.

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For the present study, 14 accessions of 8 species of the genus *Cajanus* were used (kindly provided by P. Gomathynayagam, Pulses Breeding Station, Tamil Nadu Agricultural University, India and L. J. Reddy, Genetic Resources Division, ICRISAT, Hyderabad, India). Among the eight species, two were Australian species and the others were Indian species. The six genotypes of *C. cajan* included five accessions and one commercially released variety (Table 1).

Genomic DNA was isolated from leaves following the CTAB method[16] with minor modifications. Five grams of tissue was ground in liquid nitrogen and suspended in 20 ml of CTAB extraction buffer containing 2% CTAB (hexadecyltrimethyl ammonium bromide), 100 mM Tris-HCl pH 8.0, 50 mM EDTA and 1% β-mercapto ethanol. The suspension was incubated at 60°C for 30 min, extracted with equal volume of 24:1 chloroform:isoamyl alcohol and centrifuged at 10,000 *g* for 10 min at room temperature. The aqueous phase was precipitated with 0.6 volume of ice-cold isopropanol and centrifuged at 10,000 *g* for 10 min at room temperature. The pellet was washed in 70% ethanol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Proteins and RNAs were removed by standard procedures[17], and the DNA was dissolved in sterile water. About 10 µg of total genomic DNA was restriction digested with 10 restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, *Bgl*I, *Pst*I, *Sac*I, *Dra*I, *Taq*I and *Sau*3AI) under the conditions specified by the supplier (Amersham, UK). The digested samples were fractionated in 0.8% or 1.3% (*Taq*I and *Sau*3AI) agarose gel in 1 × TAE buffer[17] at a constant voltage of 5 V/cm. The DNA was Southern transferred[18] onto nylon membrane (Hybond N^+, Amersham, UK) and hybridized to the probes pTA71and Ver 18-6. pTA71 contained an 8.95 kb *Eco*RI fragment of full-length nuclear rDNA repeat unit of wheat[19], and Ver 18-6 contained a 3.7 kb *Eco*RI fragment including the intergenic spacer (IGS) region of *Vicia faba*[20].

Length of rDNA repeats units was determined with reference to 1 kb ladder (Gibco-BRL, USA) and λ phage DNA digested with *Hin*dIII (Sigma) as markers. RFLPs observed in the eight species for the rDNA probes were scored for presence/absence, ignoring the intensity of the fragments. A few hazy fragments wherever observed were not included in the data analysis. Similarity index in all pair wise combinations was calculated as $2m_{xy}/(m_x + m_y)$, where m_{xy} was the number of fragments shared by two species and m_x and m_y were the number of fragments in each species. Phylogenetic relationship based on percentage similarity was established by constructing a dendrogram using MultiVariate Statistics Package[21] following unweighted pair group with arithmetic average (UPGMA) method[22].

Genomic DNA digested with *Eco*RI and hybridized to pTA71 showed 2 bands (about 8 kb and 3 kb) in all species except *C. cajan* in which three bands of about 6 kb, 3 kb and 2 kb length were observed. The same DNA when probed with Ver 18-6 hybridized only to the 3 kb and 2 kb fragments indicating its specificity to the flanking and intergenic spacer regions of the rDNA repeat units. However, digestion with the other six base pair-recognizing restriction enzymes *Dra*I, *Hin*dIII, *Bgl*I, *Bam*HI and *Pst*I showed identical patterns when hybridized to both pTA71 and Ver 18-6. Digestion with *Dra* I showed a single fragment of about 22 kb size in *C. cajan*, *C. reticulatus*, *C. mollis* and *C. albicans*, and a fragment of about 20 kb in *C. scarabaeoides* and *C. platycarpus*. This indicates that the rDNA repeat unit in these species has no sites for *Dra*I. In *C. goensis* there were two fragments of about 3 kb and 2 kb length. In *C. lineatus*, a prominent band of 4.3 kb and two hazy fragments of about 5 kb and 6 kb length were observed (Figure 1). The hazy fragments could be artefacts, or could have arisen through methylation of *Dra*I sites preventing cleavage[23,24]. Digestion with *Hin*dIII showed a single fragment of more than 15 kb length in *C. albicans*, *C. mollis*, *C. reticulatus* and *C. cajan*. In *C. platycarpus* and *C. goensis* a single band of 14 kb and 11 kb length, respectively was observed. In *C. scarabaeoides* and *C. lineatus* two bands of 8 kb and 7 kb were observed. Digestion with *Bgl*I and *Bam*HI showed 3–4 and 4–5 bands in each species, respectively. *Pst*I showed 4 bands in all the species except *C. lineatus* in which three bands were observed.

Restriction digestion with *Eco*RV and hybridization to both pTA71 and Ver 18-6 showed a single DNA band in all the species indicating that the rDNA repeat unit may be flanked by the recognition sequence for this enzyme (Figure 2). The rDNA repeat unit length as estimated from the *Eco*RV digested DNA hybridized to

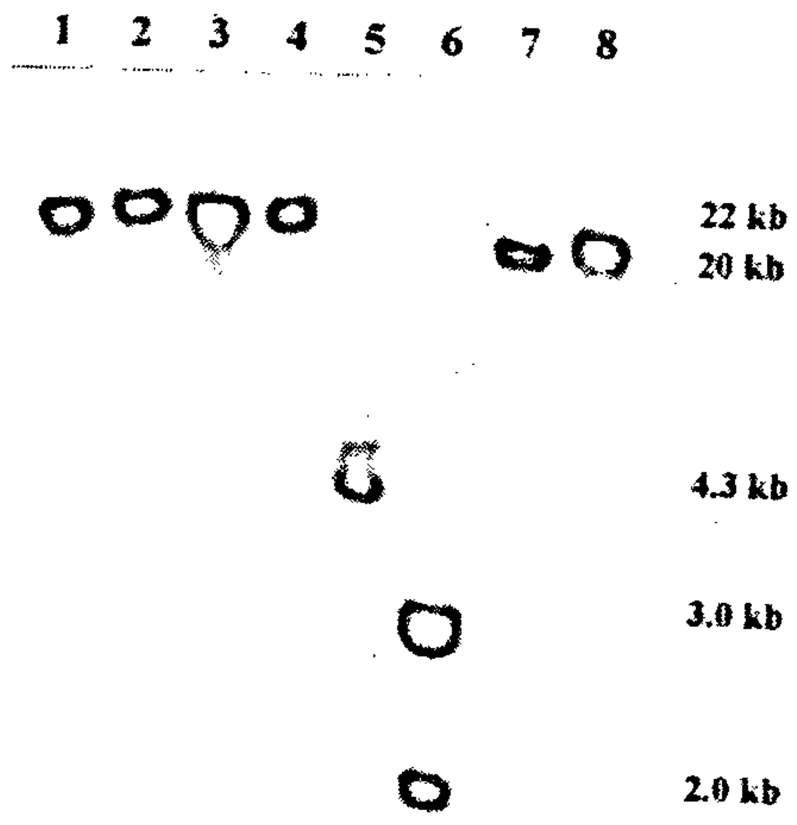

Figure 1. Restriction digestion of genomic DNA from 8 species of *Cajanus* (Serial nos 1 to 8 in Table 1) with *Dra*I and hybridization to pTA71.

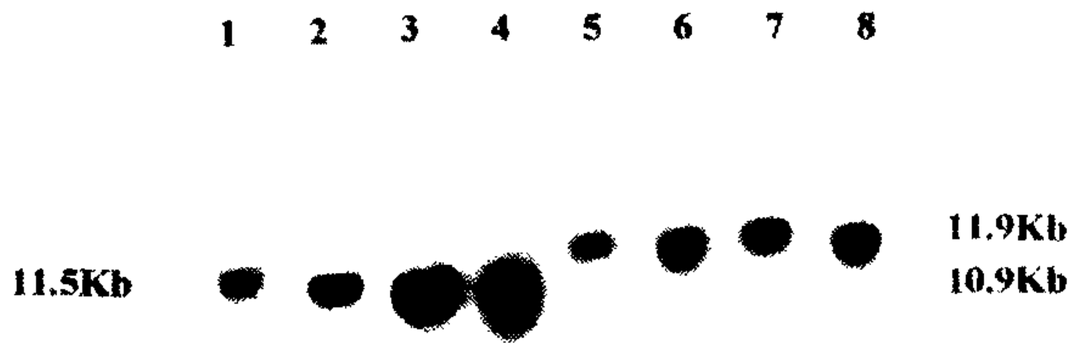

Figure 2. Restriction digestion of genomic DNA from 8 species of *Cajanus* (Serial nos 1 to 8 in Table 1) with *Eco*RV and hybridization to pTA71.

pTA71 was 11.5 kb in *C. platycarpus*, 10.9 kb in *C. scarabaeoides*, *C. goensis* and *C. lineatus*, and 11.9 kb in *C. albicans*, *C. mollis*, *C. reticulatus* and *C. cajan*. As the RFLP with *Eco*RI indicated the presence of one or two internal sites for the enzyme, we have carried out double digestion with *Eco*RI and *Eco*RV. The sum of the restriction fragments observed after hybridization to pTA71 was almost equal to the size estimated using the DNA digested with *Eco*RV alone.

As the rDNA polymorphism in *Cajanus* revealed by six base pair-recognizing enzymes was less, for increasing the resolution, the samples were digested with four base pair-recognizing enzymes. Ribosomal DNA has many sites for these enzymes both in the coding as well as in the spacer regions[20,25]. Ribosomal DNA for *Cajanus* species showed 8 bands when digested with *Sau*3AI and hybridized to pTA71 (Figure 3). DNA digested with *Taq*I showed four fragments which were less than 1 kb.

The cultivated species *C. cajan* exhibits a great amount of variation for several morphological traits[26–28]; yet it showed very little variation in nuclear RFLP[7]. Though rDNA polymorphism at an intra-specific level would not be profound, considerable amount of polymorphism within a species has been observed particularly with four base recognizing enzymes and probes specific to IGS regions of rDNA[24]. However, the six genotypes of *C. cajan* included in the present study did not show polymorphism in any of the enzyme–probe combinations despite the fact that pigeonpea is one of the exceptions in grain legumes that has a tendency towards ourcrossing[29].

The RFLPs observed in the eight species detected by the ten enzymes and two probe combinations were scored for presence and absence and were used to analyse the genome relationships among the species. The similarity index between the species varied from 0.95 to 0.53. A dendrogram constructed based on the similarity

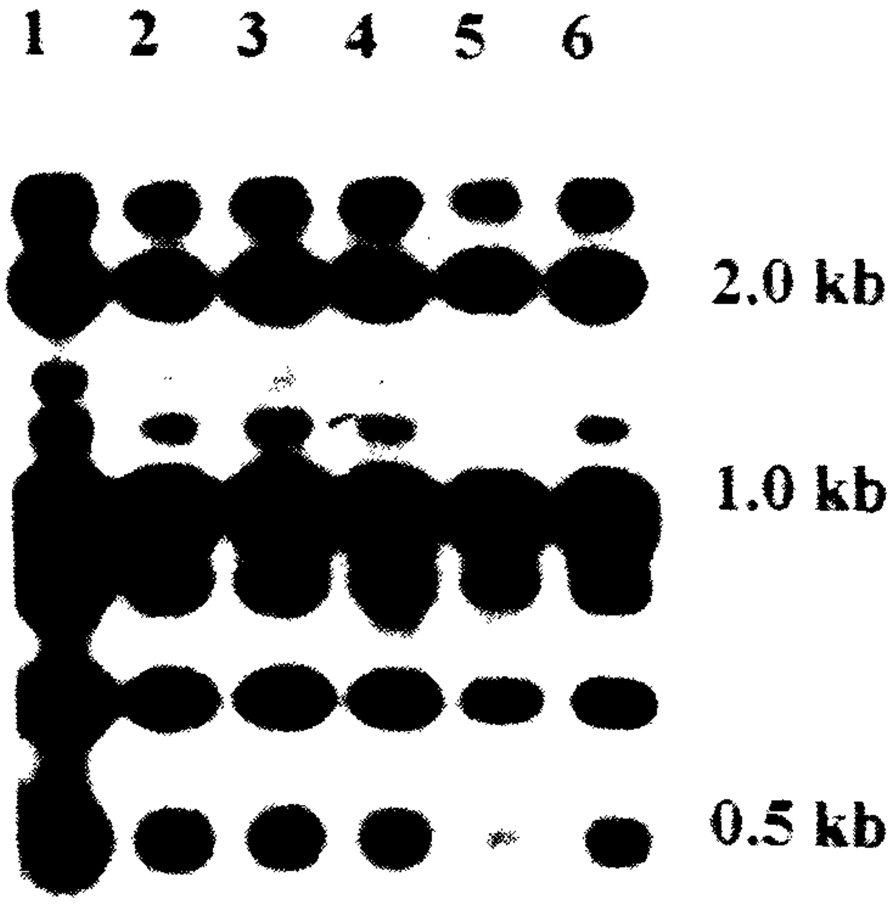

Figure 3. Restriction digestion of genomic DNA from 8 species of *Cajanus* (Serial nos 1 to 6 in Table 1) with *Sau*3AI and hybridization to pTA71.

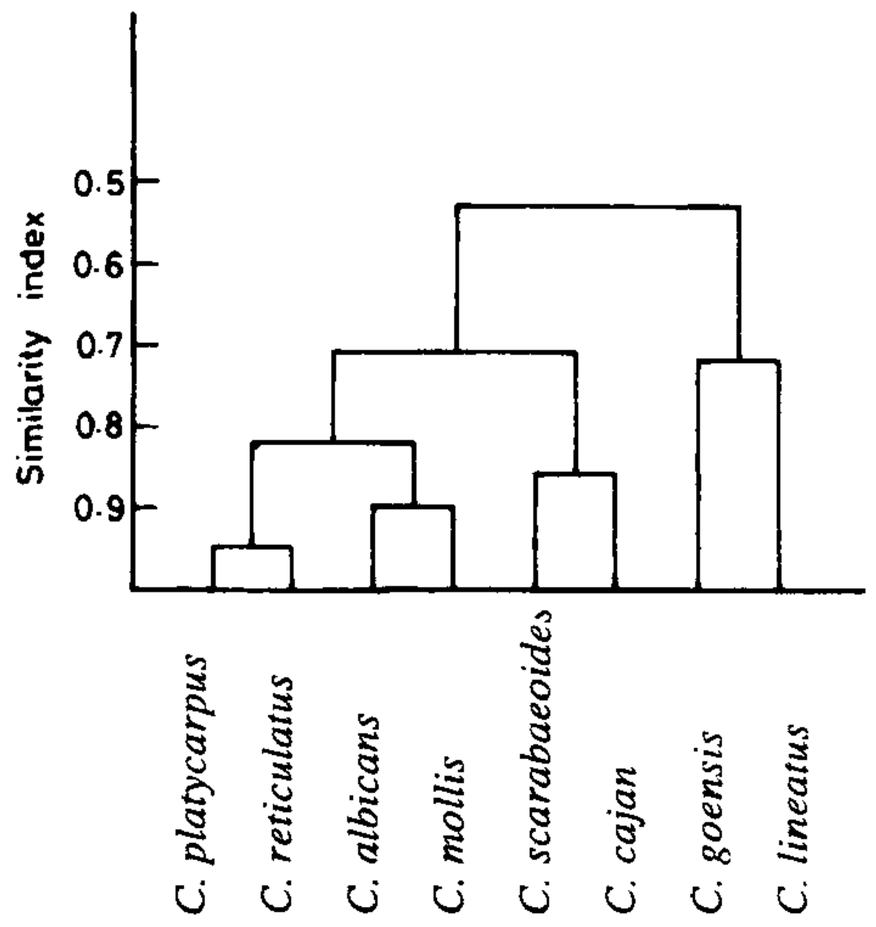

Figure 4. Cluster diagram based on similarity index showing genomic relationship between cultivated and wild species of *Cajanus*.

index revealed interesting features of genome relationship between *C. cajan* and its wild relatives (Figure 4). Currently there are about 32 species in *Cajanus*, 18 of which are endemic to Asia (mostly India), 13 to Australia and 1 to western Africa[28]. Inter-specific hybridization in *Cajanus* has very often produced fertile hybrids[30] with variable degree of crossability and fertility. This variation has not been specifically related to the place of origin of the species involved in the hybridization.

Based on RAPD studies it has been reported that the Australian species *C. acutifolius*, *C. reticulatus* and *C. grandifolius* were less closely related to *C. cajan* and other Indian species. However, the present study revealed very close relationship between the Australian species *C. reticulatus* and the Indian species *C. paltycarpus* (95% similarity). The Australian species *C. scarabaeoides* showed 86 per cent similarity with the Indian species *C. cajan*. A close genetic relationship between these two species has also been reported earlier[5,7]. Therefore, available data point out that it cannot be generalized that the species from the two continents are less closely related.

Earlier studies have clearly established *C. cajanifolius* as the progenitor of cultivated *C. cajan*[1,7,27,28]. The popular hypothesis is that *C. cajan* could have evolved through a series of gene mutations in *C. cajanifolius*[5]. The alternate hypothesis is that it could have evolved from natural inter-specific hybridization of *C. lineatus* with *C. scarabaeoides*. The present study based on rDNA analysis showed a close relationship between *C. cajan* and *C. scarabaeoides* (86% similarity) as observed from the morphocytological and electrophoretic data[5] and also from the RFLP data[7]. In fact, genetic distance between *C. cajan* and *C. scarabaeoides* was lesser than that between *C. cajan* and *C. cajanifolius*[7]. *C. scarabaeoides* is the most widely distributed wild species among all species of *Cajanus*, and its hybrids with *C. cajan* are highly fertile with normal meiosis[28,30]. However, our data did not show close relationship of *C. lineatus* either with *C. cajan* or *C. scarabaeoides*. We have observed a distant relationship of *C. lineatus* with all the other species studied, except *C. goensis* (Figure 4). Therefore, molecular data available to date do not support polyphyletic origin of *C. cajan* through natural hybridization between *C. lineatus* and *C. scarabaeoides*.

The present study has revealed the length of rDNA repeat unit in some species of *Cajanus* and helped to analyse the genome relationships among them based on the restriction site variations in rDNA. The results have added further information about the genome relationships and about the intra-specific variation in the cultivated species which could be useful to plant breeders to exploit the wild germplasm. Experiments on mitochondrial and chloroplast genome of *Cajanus* and other related genera such as *Dunbaria, Rhynchosia* and *Flemingia* from all the three centres of origin are being carried out to further resolve the genetic relationship and origin of the cultivated species *C. cajan*.

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