THE USE OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FOR THE STUDY OF GENETIC DIVERSITY IN *CAJANUS CAJAN*

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ABSTRACT

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is one of the major pulse crops of the tropics, and is important in small-scale farming in mainly developing, semi-arid regions. Random amplified polymorphic DNA (RAPD) was employed to assess the genetic diversity within and between 27 accessions of pigeonpea available at ICRISAT. The 25 random decamer primers used, generated between 4 and 14 amplification products, which had a size range from 0.29 kb to 2.0 kb. All the accessions analysed showed low levels of polymorphism. This result indicates that RAPD markers are not appropriate to assess the genetic variation within and between accessions of pigeonpea.

INTRODUCTION

Cajanus cajan is one of the most important pulse crops of the tropics. Its world production is about 2 million tons annually, with India contributing more than 90% (Mueller *et al.*, 1990). Seed production among legumes ranks fifth in area and fourth in production after beans, peas and chickpeas, but it is used in more diverse ways than other crops (van der Maesen, 1990).

Research on pigeonpea started in India, where there is a high diversity of cultivars, which are a result of selection by farmers over the centuries. Breeders selected from local segregating populations to produce large-seeded pigeonpeas for direct consumption, but were not very successful. Since then, attempts to improve pigeonpea have been inadequate and it is expected that large genetic gains will be achieved, if adequate attention is given (Byth *et al.*, 1981). For the improvement of pigeonpea, germplasm is the basic material, and the key to success lies in the crop s genetic diversity (ICRISAT, 1982b). The measurement of genetic diversity is useful in assisting breeders in the procedure of selecting improved varieties. Selecting for the optimal expression of a desired characteristic is an enormous task but can be facilitated by the use of markers.

Since the 1980's, molecular markers have been used extensively. One advantage of using these markers is that in analysing variation within the genetic material itself, one is avoiding difficulties with differential gene expression due to developmental state or environmental influences (Newbury *et al.*, 1997). More recently, random amplified polymorphic DNA (RAPD) generated by the polymerase chain reaction (PCR) has been used effectively to understand the extent and distribution of the genetic variation available within germplasm, and to identify markers for breeding. Examples of this include *Musa* (Howell *et al.*, 1994), *Brassica oleracea* (Kresovich *et al.*, 1992), *Sorghum* (Pammi *et al.*, 1994), *Vicia sativa* subsp. *sativa* (Saidi, 1997) and *Oryza glumaepatula* (Buso *et al.*, 1998).

The RAPD technology introduced by Williams *et al.* (1990) is a variation of the standard PCR, in which single oligonucleotides of arbitrary sequence are used for the random amplification of random DNA segments from single individuals.

Currently only a few studies have been performed on *Cajanus cajan* at a molecular level. Therefore, a range of pigeonpea accessions was used in this study to demonstrate the applicability of RAPD analysis for assessing the genetic diversity between and within accessions of *Cajanus cajan* germplasm. Genetic polymorphisms revealed within the material should be able to be used for the identification of markers that could be useful for future genetic mapping and improvement programmes.

MATERIALS AND METHODS

Plant material

Twenty-seven accessions of *Cajanus cajan*, listed in Table 1, were provided by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (India) for the study. These accessions were from various geographic regions, including India, Trinidad, Tobago, Sri Lanka, Puerto Rico, Russia, Kenya and Australia. Seeds were sown in jiffy pots containing sterilised moist compost. The pots were then placed in a greenhouse that was maintained at 27°C. Three weeks after sowing, three plants of each accession were sampled to provide material for DNA extraction.

DNA ISOLATION

DNA was extracted from 20 mg (fresh weight) of leaf tissue from the edges of young leaves following the protocol of Gawel and Jarret (1991) with minor modifications. Leaf material was placed in a 1.5 ml microfuge tube and ground up in liquid nitrogen using a polypropylene pellet pestle mixer driven by a hand-held cordless motor (Kontes, U.S.A.). The homogenate was mixed with 700 µl of extraction buffer (6.055g Tris, 40.91g NaCl, 3.722g EDTA $(Na)_2$, 20g CTAB, 429.31 ml H_2O, 10 µl 0.2 % β-mercaptoethanol) that had been kept at 65°C for an hour before 600 µl of chloroform:isoamyl alcohol (24:1) was added. After mixing by inversion for five minutes at room temperature, the mixture was centrifuged for five minutes at 13000 rpm. The aqueous layer was recovered, mixed with an equal volume of ice-cold isopropanol and then further mixed by inversion quickly and left for five minutes in a fridge. The nucleic acid precipitate was recovered by centrifugation at 13000 rpm, washed with

ICRISAT Accession Number	Random Number
NSSL 73128.01	1
ICPL 88009	2
ICPL 88034-3	3
ICPL 90004-1	4
ICPL 86012	5
ICP 28	6
ICP 6914	7
ICP 6973	8
ICP 6997	9
ICP 26	10
ICP 6932	11
ICP 9150	12
ICP 7035	13
ICP 10898	14
ICP 7263	15
ICP 7104	16
ICP 7118	17
ICP 7143	18
ICP 8006	19
ICP 6443	20
ICP 8077	21
ICP 7213	22
ICP 7631	23
ICP 4023	26
ICP 6971	27
ICP 7001	28
ICP 9122	29

Table 1. List of *Cajanus cajan* germplasm used in the study

300µl of 70 % ethanol and re-centrifuged for five minutes at 13000 rpm. The resulting pellet was dried in a vacuum-desiccator for ten minutes, and re-suspended in 100µl of sterile distilled water to produce the DNA stock solution. The tubes were then put into a fridge (37°) for one hour. The concentration of DNA was estimated by running the samples on 0.8% agarose gels in TBE buffer (Sambrook *et al.*, 1989), staining with ethidium bromide, and visual assessment of band intensities compared with lambda DNA standards.

Polymerase chain reaction

A set of 23 decanucleotides (OPC-20, OPN-11, OPM-14, OPG-14, OPG-18, OPO-17, OPK-19, OPB-17, OPO-15, OPM-07, OPN-02, OPAX-04, OPY-01, OPAA-01, OPX-04, OPH-12, OPAT-01, 0PAT-03, OPAT-06, OPAT-08, OPX-01, OPX-15, OPX-16) purchased from Operon Technologies (Alameda, California) were employed for PCR amplification following the method used for rice (Virk *et al.*, 1995). Amplification reactions were carried out in 25 µl volumes containing approximately 20 ng of template DNA, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.4 µM concentration of a single decanucleotide, 2.5 mM $MgCl_2$, 1.0 U *Taq* polymerase (Bioline) and 1 x ammonium incubation buffer (from manufacturer). Each reaction mixture was mixed gently prior to adding two drops of light mineral oil.

DNA amplification reactions were performed in a Hybaid-Omnigene thermocycler. The cycling regime was as follows: one cycle of two min at 95°C; two cycles of 30 sec at 95°C, one min at 37°C and two min at 72°C; two cycles of 30 sec at 95°C, one min at 35°C and two min at 72°C; 41 cycles of 30 sec at 94°C, one min at 35°C and two min at 72°C. The last cycle was followed by a final incubation for five min at 72°. Amplification products were analysed by gel electrophoresis in 1.0% agarose gels in 1 x TBE buffer (Sambrook *et al.*, 1989), and DNA fragments were stained with ethidium bromide. Gels were photographed under UV light using the Digital Imaging System (Alpha Innotech Corporation).

Data analysis

For the analyses only primers giving scorable patterns for all genotypes were considered. DNA fragments detected after electrophoretic separation were visually scored. Specific amplification products were scored as present or absent.

RESULTS

Optimization of RAPD protocol

The sensitivity of RAPDs to changes in experimental parameters is well known (Munthali *et al.*, 1992), and therefore several experiments were carried out to establish a protocol resulting in reproducible results and to confirm the necessity of using exactly the same protocol to get a high level of reproducibility. To optimize the PCR amplification conditions for pigeonpea, experiments were performed with varying concentrations of DNA template, *Taq* polymerase and primer. Among the parameters examined, DNA template concentration was found to be very important for successful amplification. At lower DNA template concentrations only a few bands were produced, whereas higher concentrations (20ng) increased the number of bands resolved. The $MgCl_2$ and dNTPs were used as optimized for the RAPD *Oryza* protocol at the University of Birmingham (Virk *et al.*, 1995).

Amplified DNA fragments from the pigeonpea accessions were observed directly. Products of the PCR amplification were compared to each other for the identification of genetic diversity within and between accessions. Depending on the primer/accession combination and the amplification conditions used, the size of amplified DNA fragments ranged from 0.29 kb to 2.0 kb. The number of amplification products resolved varied between four and 14. Typically, two to five major fragments and a number of bands of lesser intensity were amplified in any given sample.

The level of reproducibility was high. Different DNA extractions of the same plant gave the same results, and the agarose gels and ethidium bromide methodologies used in the study produced reproducible banding patterns.

Variation within and between accessions

Random amplified polymorphic DNA analysis was performed using DNA samples from three individual plants of 27 accessions of pigeonpea (Table 1) and employing 25 decanucleotides of arbitrary sequence. All amplifications were found to be reproducible when repeated on different occasions.

The use of all primers resulted in amplified DNA fragments, but primers OPN-11, OPM-14, OPG-14, OPG-18 and OPK-19 resulted in a large number of weakly stained bands, and primer

OPO-15 gave banding patterns showing poor reproducibility when used at the routine concentration of 0.4μM. For the primers resulting in faint bands, reproducible and easy scorable amplification products were obtained by an increase in primer concentration from 0.4 to 0.8μM. However, primer OPO-17 showed poor reproducibility, even when the primer concentration was increased.

Among the whole material studied, low levels of polymorphism were found with the 25 primers used. No differences were found within any of the 27 accessions of pigeonpea (Figures 1 and 2). Amplification products using DNA extracted from three individual plants from each accession yielded no polymorphic bands. Similarly, low levels of polymorphism were detected between the accessions. Only one out of the 25 primers used, yielded two reproducible polymorphic bands. Amplification products generated with OPH-12 are detected between 0.5 kb and 1.6 kb. Since the genotypes were monomorphic for nearly all bands, data derived from this study could not be used for subsequent analysis.

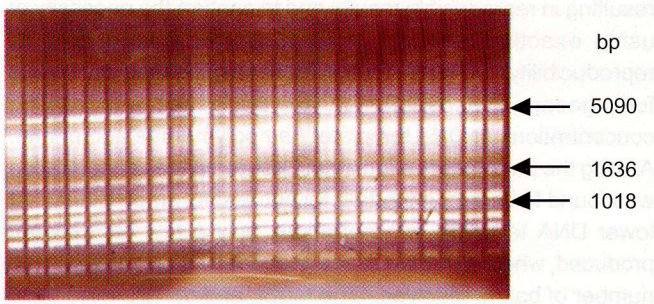

Figure 1. Lanes representing amplification products of genomic DNA extracted from three individuals of seven accessions of pigeonpea using primer OPAX-04. Left to right: genotypes with random number 1-8 (Table 1).

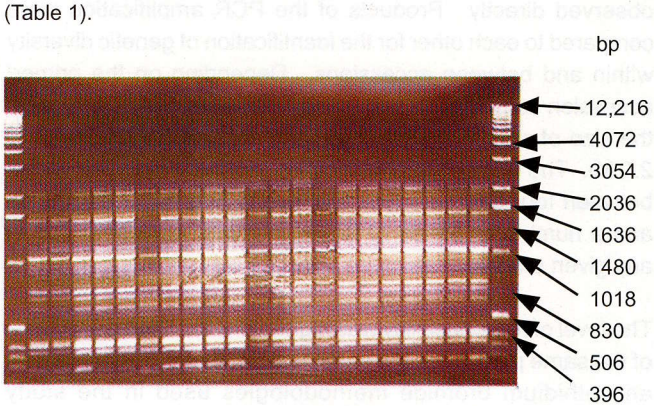

Figure 2. Amplification products using DNA extracted from three individual plants of six accessions of pigeonpea using primer OPH-12. Lane 1 and 20, 1 Kb ladder. Lanes 2-4, NSSL 73128.01. Lanes 5-7, ICPL 88009. Lanes 8-10, ICPL 88034-3. Lanes 11-13, ICPL 86012. Lanes 14-16, ICP 28. Lanes 17-19, ICP 6973.

DISCUSSION

Applicability of RAPDs for diversity studies in pigeonpea

A primary goal of the study was to demonstrate the applicability of RAPD markers for assessing the genetic diversity within and between accessions of pigeonpea. The results demonstrate that RAPD markers are not extremely useful to identify DNA polymorphisms in pigeonpea. This is because the diversity within this species is relatively low, which means that not enough polymorphic bands can be detected for subsequent data analysis. Only a single primer out of the 25 decanucleotide primers used was able to discriminate between accessions included in the present study.

In terms of cost, speed of data generation and simplicity, RAPD is superior to other techniques. RAPDs only involve PCR and agarose gels, and polymorphisms are usually visualized without the need of labelled radioisotopes. However, one problem encountered with the technique for the study of genetic diversity in pigeonpea is the dominant expression of the majority of the markers, where segments of DNA of the same length are either amplified or not from individual plants. Since the pigeonpea accessions were genetically quite similar, the inadequacy of RAPD markers to distinguish homozygotes from heterozygotes reduces the efficiency of the technique.

Further, reproducibility of the marker is dependent upon the annealing of the primers. Primers with a high GC composition anneal at a higher temperature than do those with a high AT composition. Therefore, it becomes more difficult to obtain repeatable results using standard PCR conditions with an increasing number of primers screened. In addition, subtle differences in dNTP concentration, Mg^{2+} concentration, cycling regime, and other conditions significantly affect the reproducibility of some RAPD analyses and the identities among some laboratories.

Recommendations for future studies

To discriminate within and between accessions of pigeonpea, a follow-up project using a different genetic marker is needed. The scope, expertise and materials exist to do this. Studies carried out at ICRISAT already showed that RFLPs and AFLPs provide good estimates of the fraction of restriction fragments which distinguish the parents of segregating populations of chickpea and pigeonpea (van Rheenen *et al.*, 1996). However, it became clear that both markers worked better for chickpea than for pigeonpea. The reason for this is not clear. Although the pigeonpea F_2 population did not, in general, show the segregation patterns expected from the parental screens, the individual F_2 plants showed essentially the same banding pattern (van Rheenen *et al.*, 1996).

It will be of great benefit to use sequence-tagged microsatellites (STMS) for assessing the genetic diversity of pigeonpea available at ICRISAT. Microsatellites are repetitive sequences consisting of di-, tri- or tetranucleotide repeats arranged in tandem arrays (Tauz, 1989). It has been estimated that, although present in much lower numbers in plant genomes when compared with animals, there are between around 5 x 10^3 and 3 x 10^5 microsatellites per plant genome (Condit and Hubbel, 1991).

Advantages of microsatellites lie in their highly polymorphic, co-dominant nature (Morgante and Olivieri, 1993). Often a large

number of alleles can be identified at a single locus and levels of heterozygosity can be detected. This makes microsatellites very useful in producing genetic maps. It is also possible to multiplex PCR reactions, using two or more sets of primers that will amplify at the same annealing temperature, or to multiplex sample loading on gels. This will improve the speed with which data can be collected.

However, unless the investigator is extremely fortunate, sequence information for DNA flanking the repeat itself is required. This information is very seldom available for the species of study, and therefore microsatellites must be retrieved first, or must be cross-transferred from other genera. The University of Birmingham has started investigations to transfer the use of microsatellite locus-specific primers from soybean to pigeonpea, but proper results have not yet been obtained.

To study genetic diversity maximally in future, informative crosses must be used to apply genetic markers successfully. The choice of crosses for mapping populations is critical, especially given the low frequency of polymorphism observed (van Rheenen, 1996). Further, more lines of pigeonpea and more primers should be used for diversity analysis. After sufficient markers have been collected, correlations in the segregation of markers and genes must be studied to establish markers for particular traits of agronomic importance.

However, many qualitative traits have low heritability, and therefore the high performance of a certain individual is no guarantee that it has desirable alleles at the relevant QTL. It will be difficult to score many phenotypes on an individual, such as disease resistance or sex-limited traits, which reveal their effects late in life or development, if many QTL are segregating and the traits are recessive. Only major genes with a high heritability should be used in future breeding programmes. But since the level of genetic variation is low in pigeonpea, it will be very difficult to select for genes such as resistance. Typically, traits closely related to fitness show lower narrow heritabilities and larger amounts of non-additive genetic and environmental variation than do traits under stabilizing conditions.

The low levels of polymorphisms observed among the accession of pigeonpea warrants the collection of germplasm from the remaining unexplored areas, which are geographically or climatically isolated from the major growing areas.

CONCLUSION

The study demonstrates that RAPD markers are not appropriate to identify DNA polymorphisms in pigeonpea. This is because the diversity within this species is low, which means that not enough polymorphic bands could be detected for subsequent data analysis. The problems encountered help to illustrate that a marker with a different genetic quality should be used in a follow-up project. Marker like sequence-tagged microsatellites offers significant opportunities to assess genetic variation in pigeonpea, because they are of a highly polymorphic, co-dominant nature.

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