Development of a DNA Extraction Protocol from *Anacardium occidentale*L. for Genetic Differentiation of Local and Exotic Germplasm

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ABSTRACT

Cashew is the most economically important nut crop in the world. Considering the present trend, future demand for this crop will be heavily dependent on exports. To meet the high export demand increased production through genetically improved planting material is needed. To facilitate the conventional breeding programs with molecular marker studies, proper DNA isolation and polymerase chain reaction (PCR) amplification method from cashew genotypes should be established. Cashew has high amount of metabolites like polyphenolic compounds, polysaccharides, tannin and RNA which interfere with DNA isolation and PCR amplification.

A simple protocol was developed for isolation of genomic DNA. This protocol involves the use of extraction buffer Tris-HCl, EDTA NaCl, SDS, β-mercaptaethanol and polyvinylpyroviridone (PVP) and subsequently two steps, Phenol : Chloroform extraction and chloroform extraction to bind and remove protein and chlorophyll. Quality and quantity of the isolated DNA was tested using restriction digestion analysis, agarose gel electrophoresis and spectrophotometry. Attempts were made to optimize the conditions for RAPD analysis. Morphological characteristics of the selected genotypes were recorded and RAPD reactions were carried out using random primers A20, A18, OPH4, OPF9, OPK12, and OPK 4.

The yield of DNA that could be extracted by the selected best method was 15 µg/mg leaf tissues. In this method there was no difference in the DNA yield in exposing the ethanol precipitation step to -20 °C overnight, but addition of PVP was a critical factor. The suitability of the DNA obtained by this method for restriction analysis was confirmed. The values of purity of DNA measured by A260/A280 vary from 1.3 - 1.8. PCR products were not observed in the RAPD assay implicating the need of further studies for optimization of conditions.

KEY WORDS: *Anacardium occidentale* L, Cashew, DNA Extraction protocol, RAPD, PVP

INTRODUCTION

Cashew is an important tropical tree belonging to the family Anacardiaceae. It is a polygamous, andro-monoceious, evergreen tropical perennial tree (Mandel, 1997). The cashew nut consists high nutrient content protein, carbohydrate, fat (mainly unsaturated fatty acids), and vitamins. Juice obtained from cashew apple made into beverage or fermented into wine. The cashew nut shell contains a viscous and dark liquid, known as cashew nut shell liquid. Cashew nut shell liquid is one of the few natural resins that is highly heat resistant and is used in braking systems and in paint manufacture (Azam-Ali, 2004). The highest production of cashew was obtained in 1993; Sri Lanka earned Rs.341.9 million from cashew exports (Surendra, 1998). The exported quantity was 66.2 MT in 2003 and 187 MT in 2004. The exported value was 42.5 Rs million in 2003 and 123 Rs million in 2004 (Anon, 2005). Considering the present trend, future demand for this crop will be heavily dependent on exports.

Several Indian varities and several indigenous types have been cultivated in the past. Indian varities are Kondachchi, Mannar, Trinidad, Batticalo, Shanthigudu, Ulal, Vital (Jayasekara, 2005). Kurunagala (7,650 ha), Puttlam (6,069 ha), Gampaha (2,100 ha) and Hambanthota (1,455) are the major cashew growing districts in Sri Lanka. Reported national average yield is approximately 4-5 Kg/tree/year, but it has potential yield of about 10-15 kg/tree/year (Jayasekara and Kodikara, 2005). One major reason for the low average yield is the use of poor planting material. There were no systematic breeding programs undertaken in Sri Lanka in the past until recently. The first three varities developed in Sri Lanka are WUCC-9, WUCC-19, WUCC-21 introduced in 2005 (Jayasekara and Jayasekara 2005).

Genetic improvement in cashew is limited by the lack of knowledge of genetic diversity of the indigenous germplasm, its seasonal behavioural pattern, perennial nature and heterogeneity. Breeding of cashew is mostly based on traditional methods of selection of useful traits, such as nut size, nut weight, sex ratio, and yield performance (Mneney *et al.*, 2001). Although classical phenotype features are still extremely useful, the efficiency of selection may be reduced by plant developmental stages or by environmental effects on measured traits. Cultivar identification is also mainly based on morphologically traits. This approach has however limited usefulness since the variation in morphological traits is influenced by environmental factors.

Many of the obstacles for cashew breeding programs could be overcome by the molecular marker technology. DNA markers play an important role to assess the genetic diversity of an existing cashew germplasm and for marker assisted selection for economically important traits. Therefore polymorphic markers are needed for identification of

varieties, and to estimate of genetic diversity among and between the populations or varieties. Random amplified polymorphic DNA (RAPD) assay is the cheapest method for identification of the genotypes within a short time period and also requires only a limited amount of DNA (Williams *et al.*, 1990). However, the basic requirement in the use of DNA markers is the availability of a reliable method for DNA extraction. Cashew is an underutilized crop which only little research have been carried out. Classical approaches in plant DNA isolation have aimed to produce larger quantities of DNA and highly purified DNA that is needed for molecular biology research.

Many simple procedures have been developed for use in preparing plant DNA for polymerase chain reaction (PCR). However, these procedures may only work for a limited number of tissue or plant species, and most are still multi-step procedures. The biochemical composition of plant tissue of different species or varities varies considerably. The chemotypic heterogeneity among species or varities may not allow optimal DNA yields from one isolation protocol, and sometimes even closely related species may require different isolation protocols (Weishing *et al.*, 1995).

In many cases, the presence of polysaccharides (e.g. starch) and secondary metabolites (e.g. phenols) often inhibit restriction endonuclease digestion and PCR amplification (Weishing *et al.*, 1995).

Many DNA isolation procedures also yielded large amount of RNA (Dodyl and Dodyl, 1990). Use of RNAase is generally accepted method to remove RNA; however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including suppression PCR amplification (Pikbart and Villeponteau, 1993).

At present molecular studies on cashew is limited. Further the high amount of secondary metabolites, orthohydroxyphenols and polysaccharides which are powerful oxidizing agents interfere with genome DNA (Nagaraja, 2000) and no simple protocol has been developed for isolation and purification of genomic DNA from cashew which is suitable for RAPD analysis.

The objective of this study is to develop a simple efficient protocol for DNA extraction and purification to investigate genetic diversity of 7 varities of cashew by using morphological characteristics and RAPD markers.

MATERIALS AND METHODS

The study was carried out at the Department of Biotechnology, Wayamba University of Sri Lanka, Makandura, Gonawila.

Plant Materials

Five trees from exotic varities and two trees from local varities, of the same age were selected from the cashew trees maintained by the Wayamba University of Sri Lanka. Fresh leaves of *Anacardium occidentale* were obtained from these trees for DNA extraction.

Morphological Characteristics

Data on morphological characteristics such as inflorescence shape, leaf apex shape, leaf margin, colour of the young leaf, shape of the cashew apple, colour of the cashew apple, and disease susceptibility (inflorescence blight) were recorded. This was recorded by visual observation of the number of inflorescences found on trees.

DNA Isolation

Two methods were tested for the DNA extraction procedure. In both methods liquid nitrogen was not used at the grinding step.

1. Phenol : Chloroform Method

Young leaf material (50mg) was ground quickly with 400 µl of extraction buffer [200mM Tris-HCl (PH 8), 25 mM EDTA (PH 8), 250 mM NaCl, 0.5 % w/v SDS, 0.1% β-mercaptaethanol]. After the addition of another 400 µl of extraction buffer, the liquid phase was transferred to 1.5 ml cold eppendorf tube. It was centrifuged at 10,000 rpm for 1 min. Supernant was transferred to another cold eppendorf tube. Equal volume of Phenol : Chloroform mixture was added by invert mixing and spun at 12000 rpm for 5 min. aqueous layer was transferred in to the new cold eppendorf tube. This was mixed well by slowly inverting the tubes with equal volume of chloroform for 3 min. and spun at 12000 rpm for 5 min. Then the supernant was transferred to another fresh tube and 1 µl of RNAase (10 mg/ml) was added to the supernant. It was allowed to stand for 15 min. in the room temperature. Two volumes of 100% ethanol were mixed with supernant. It was kept for 3-5 min. in the room temperature. It was spun at 12000 rpm for 10 min. The DNA pellet was washed twice with 70% ethanol for 4 minutes at 12000 rpm and dried at room temperature. The DNA pellet was resuspended in 30 µl of autoclaved distilled water and stored at - 20 °C.

This protocol was tested with the presence and absence of PVP in the extraction buffer, with and without -20 °C storage overnight for DNA precipitation.

2. CTAB Method

Young leaf material (50 mg) was ground quickly with 300 µl of extraction buffer [50mM Tris-Hcl (PH 8), 10mM EDTA (PH 8), 0.7mM Nacl, 2 % CTAB, 0.1 % β-mercaptaethanol and 2.5 % PVP]. After the addition of another 300 µl of extraction buffer, the liquid phase was transferred to 1.5ml cold eppendorf tube. It was warmed in a 60°C water bath for 15 minutes. 500µl of chloroform was added and centrifuged at 13000 rpm for 10 minutes. 1 µl RNAase was added to supernant and it was incubated at 37°C for 15 minutes. Two volumes of 100% ethanol were added and centrifuged at 13000

rpm for 10 minutes. The DNA pellet was washed twice with 70% ethanol for 4 minutes at 12000 rpm and dried at room temperature. The DNA pellet was resuspended in 30 µl of autoclaved distilled water and stored at - 20 °C.

Quantification of Isolated DNA

DNA concentration was determined by measurement of optical density. For this, 15µl of DNA was diluted with 2985 µl of sterile distilled water and the optical density was measured at 260 nm against sterile distilled water blank in a UV-visible spectrophotometer. The DNA concentration was measured at 260 nm. Optical density was also taken at 280 nm. Total DNA purity was tested by taking the ratio of OD values at 260:280.

Quality of Isolated DNA for Restriction Digestion

10 µl of DNA were digested in 3 µl of buffer, 1µl of BSA, 1µl of enzyme (*Eco*R1) 20 µl of distilled water. All the components were mixed in a microfuge tube and it was placed in a water bath at 37 °C for 2 hours to complete digestion. Finally the reaction mixture was heated at 65 °C for 10 minutes. Digested cashew DNA was run in an agarose gel.

Polymerase Chain Reaction

The concentrated DNA samples were diluted to give 10^{-1} and 10^{-2} dilutions. Five random primers A20, OPH4, OPF9, OPK12 and OPK 4 were used for PCR. The DNA amplification reactions were performed in a volume of 20 µl containing 5 µl genomic DNA from the diluted samples. 0.8µl of (5mM) dNTP mixture (dATP, dGTP, dCTP, dTTP), 0.75 µl of primer, 0.35 µl of Taq polymerase (5U/µl), 1.8 µl of $MgCl_2$ (25mM), 2 µl of buffer (Mg free-10x) and 9.3 µl of autoclaved distilled water.

The mixture was gently mixed. The amplification was performed in a Thermocycler for 45 cycles, each cycle consisting of 90 °C for 1 minute, 36 °C for 1 minute and 72 °C for 2 minutes. Amplification products were loaded in 1 % agarose gel for electrophoresis in 0.5xTBE buffer and visualized by ethidium bromide staining on a UV transilluminator.

RESULTS AND DISCUSSION

Morphological Studies

Six morphological characteristics of seven genotypes of cashew were studied (Table 1). There were no differences within the exotic varities and local varities but differences of the morphological traits were found between exotic varities and local varities as determined by visual observation. Clear differences were observed with respect to the shape of the inflorescence, colour of the cashew apple and shape of the cashew apple between the exotic and local genotypes accessions. E4 and E5 genotypes of the exotic accessions showed susceptibility to inflorescence blight.

DNA Extraction

It has been reported that the isolation of genomic DNA and subsequent PCR amplification of cashew DNA to be complicated due to abundance of polyphenolics, RNA and other secondary products (Nagaraja, 2000). Polyphenolics and other secondary plant compounds, cause damage to DNA and /or inhibit restriction enzymes and Taq polymerase (Doyle and Doyle, 1990).

No DNA yield was obtained with CTAB protocol. Similar results were obtained from the standard Phenol : Chloroform method. The experiments were repeated four times. However, an appreciable amount of DNA was able to isolate from the Phenol : Chloroform method when PVP was used at the grinding stage of leaf tissue (Figure 1). PVP and β-mercaptaethanol help to remove polyphenolics (Wendal, 1990). The addition of PVP has been used

Table 1 - Six morphological characteristics of seven varities of cashew:

Genotype	Inflorescence shape	Leaf apex shape	Young Leaf colour	Cashew apple colour	Cashew apple shape	Disease susceptibility of the inflorescence blight %
E 1	B	R	L	Re	A	10
E 2	B	R	D	Re	A	10
E 3	B	R	D	Re	A	10
E 4	B	R	D	-	-	95
E 5	B	R	D	-	-	85
L 1	P	R	L	Y	F	20
L 2	P	R	Re	Y	F	10

E1, E2, E3 E4, E5- Exotic genotypes
B- Broadly pyramid
P- Pyramid (Deltoid)
L1, L2 - Local genotypes
L- Light brown
D- Dark brown
R- Rounded
Re- Red
Y- Yellow
A- Angular
F- Flattened

to isolate genomic DNA from the polyphenol rich plants such as cotton (Chaudhry *et al.*, 1999). This protocol involves use of Phenol : Chloroform and Chloroform extraction steps initially, to precipitate protein and chlorophyll. No difference of DNA yield was observed with and without overnight incubation at -20 °C at DNA precipitation.

The modified protocol developed in this paper was used for isolation and purification of DNA. The result indicated that the average yield of genomic DNA was about 15 µg /mg of leaf tissue (Table 2). Cashew DNA yield is relatively poor than other crops like rubber, rice and coconut.

The undigested sample showed a high-molecular weight band and *Eco*R-1 treated sample migrated further than the undigested sample and developed a smear, indicating that the purity of DNA is sufficient for PCR reactions (Figure 2). Purity values of cashew DNA were taken in each genotype at 260:280 ratios of OD values (Table 3).

Results show that the isolated DNA by this method to be intact, pure and with enough concentration for further studies.

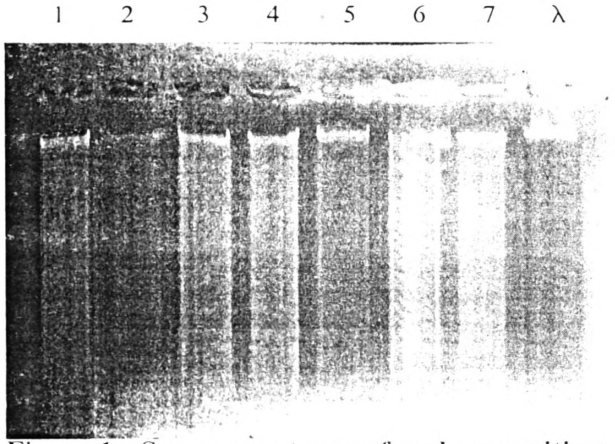

Figure 1 - Seven genotypes of cashew varities:
1, 2 - local varities
3, 4, 5, 6, 7 - Exotic varities

Table 2 -DNA yield by each method:

Method	DNA (µg/mg)
CTAB	No DNA
Phenol : Chloroform (with PVP)	15 µg/mg of leaf tissue
Phenol : Chloroform (without PVP)	No DNA

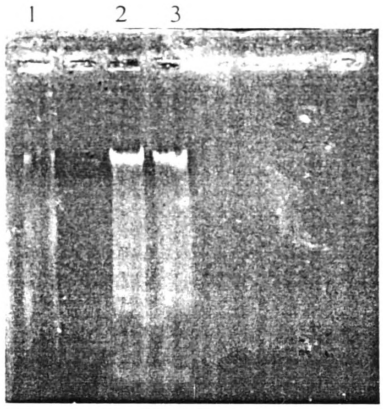

Figure 2 - Gel photograph of Cashew digestion:
1-Digested cashew DNA
2, 3 - Undigested cashew DNA

Table 3 - Purity value of cashew DNA:

Genotype	Purity value (260/280)
E 1	1.30
E 2	1.62
E 3	1.80
E 4	1.57
E 5	1.48
L 1	1.32
L2	1.59

Further, the isolated genomic DNA was tested for PCR amplification reactions by changing concentrations with the primers A18, A20, OPF9, OPH4, OPK12, and OPK4. Amplified products were not observed for the tested DNA concentrations of 10^{-1} and 10^{-2} for PCR reactions. In this experiment only the different concentrations of DNA was tested for RAPD. It is possible that the selection of primers, $MgCl_2$ concentration and DNA quality may affect the amplification. Rubber DNA which was used as the positive control was amplified in this experiment. It was successful with the A20 primer.

CONCLUSION

The identification of genotypes within the local accessions and exotic accessions was not possible by the morphological characters studied. A simple DNA extraction protocol was developed to isolate DNA from cashew. PVP was found to be an essential ingredient in the cashew DNA extraction protocol. Overnight incubation at -20 °C at the DNA precipitation stage did not improved the yield of the DNA. The genetic differentiation of seven varities was not possible using RAPD screening. PCR conditions for RAPD analysis should be optimized. Further improvements of the PCR conditions have to be considered in the future experiments.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Miss Nimali de Silva, Assistant lecturer and other staff members of Department of Biotechnology, Wayamba University of Sri Lanka. Sincere thanks are extended to Mr. K Yakandawala, lecturer of Department of Horticulture and Landscape Gardening and Mr. K.I Karunarathna, Computer instructor, Computer Unit, Wayamba University of Sri Lanka, Makandura, Gonawila, Sri Lanka.

REFERENCES

Anon, (2005). Plantation sector statistical pocket book, planning division, ministry of Plantation Industries, Colombo, pp.167-172.

Azam-Ali S. H. and E. C. Judge, (2004). "Small-scale cashew nut processing." Online. Available at http://www.fao.org/inpho/content/documents/vlibrary/ac306e/ac306e04.htm. [Retrieved June 2006].

Chaudhry, B., A.Yasmin, T.Husnain, and Reazuddin. (1999). "Mini-Scale genomic DNA extraction from cotton."*Journal of plant Molecular biology*17, 1-7

Doyle, J. J., and J. L. Doyle. (1990). "Isolation of plant DNA from fresh tissue." Focus 12, 13-15

Jayasekara, S. J. B. A., and P. B. Kodikara. (2005). "The cashew nut." In S. J. B. A. Jayasekara and N. E. M. Jayasekara (eds.), *Cashew cultivation principals and techniques* (pp.1-13). Sri Lanka cashew cooperation and Wayamba university of Sri Lanka.

Jayasekara, N. E. M., (2005). "Cashew taxonomy, Botany, Crop improvement and varities." In S. J. B. A. Jayasekara and N. E. M. Jayasekara (eds.), *Cashew cultivation principals and techniques* (pp. 34-41). Sri Lanka cashew cooperation and Wayamba university of Sri Lanka.

Mandel, (1997). *Cashew Production and Processing Technology*, India, pp.1.

Mneney, E. E., S. H. Mantell, and M. Bennet. (2001). "Use of Random amplified polymorphic DNA (RAPD) markers to reveal genetic diversity within and between populations of cashew." *Journal of Horticultural Science Biotechnology* 76,375-383.

Nagaraja, K. V. (2000). "Biochemical composition of cashew (*Anacardium occidentale* L.) kernel testa." *Journal of Food science and Technology* 37, 554-556.

Pikbart, M. J., and B. Villeponteau. (1993). "Suppression of PCR amplification by high levels of RNA." *Journal of Biotechniques* 14, 24-25.

Surendra, G. B. B. (1998). "Integrated production practices of cashew in Sri Lanka." Online. Available at http://www.fao.org/docrep/005/ac451e/ac451e08.htm [Retrieved March 2006].

Weishing, K., H. Nybom, K.Wolff, and W.Meyer, eds. (1995). *DNA finger printing in plants and fungi.* CRC Press, Boca Raton, Florida, USA.

Wendal, J. F. (1990). "Visualization and interpretation of plant isozymes." In D. E Soltis and P. S Soltis (eds.), *Isozymes in plant biology* (pp. 8). Chapman and Hall.

Williams, J. G. K., A. Kubelik, K. J. Livak, J. A. Rafiski, and S.V. Tingi. (1990). "DNA polymorphisms amplified by arbitrary primers are useful as genetic markers." *Journal of Nucleic Acids Research* 18, 6531-6535.