Comparison of Effectiveness of RAPD, ISSR and SSR Markers for Analysis of Coconut (*Cocos nucifera* L.) Germplasm Accessions

R. Manimekalai, P. Nagarajan and P.M. Kumaran[1]

Crop Improvement Division
Central Plantation Crops Research Institute
Kasaragod - 671 124
Kerala, India

ABSTRACT. *The effectiveness of Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) markers was investigated to identify polymorphism among worldwide collection of coconut germplasm accessions. RAPD (ten primers), ISSR (ten primers) and SSR (ten primer pairs) techniques produced 86, 97 and 92 polymorphic markers, respectively in 33 coconut germplasm accessions. RAPD and ISSR produced 76.7% and 82.9% polymorphic markers, respectively whilst SSR markers exhibited 100% polymorphism. Mean Polymorphism Information Content (PIC) value was highest for SSR (0.78). ISSR markers exhibited relatively high PIC (0.31) compared to RAPD markers (0.23). SSR markers exhibited a wide range of similarity (0.000-0.857) compared to RAPD and ISSR markers. Between the two multilocus markers viz., RAPD and ISSR, the latter was more efficient based on PIC value, percentage of polymorphism and better reproducibility. The Mantel test gave r=0.510 between RAPD and ISSR similarity matrices revealed high correlation between them. There was no correlation between RAPD:SSR and ISSR:SSR similarity matrices. As there was a good correspondence between RAPD and ISSR similarity matrices, ISSR may be used as an alternative to replace RAPD in the genetic diversity assessment.*

INTRODUCTION

Coconut (*Cocos nucifera* L.) is the most extensively grown and used nut in the world, playing a significant role in the economic, cultural and social life of over 80 tropical countries. In India, it is grown in 1.9 million ha and production is 9.5 million nuts (FAOSTAT, 2004). Improvement of this crop is a strategically long-term process. Markers based on differences in DNA sequence between individuals generally detect more polymorphism than morphological and protein based markers (Tanksley *et al*., 1989). Molecular markers play a vital role in easing the breeding processes aiming for the heterosis.

Molecular marker techniques have been proven powerful in estimation of genetic diversity. Thus, PCR based multiple loci marker techniques which include Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR), and Simple Sequence Repeat (SSR) or microsatellites (Gupta and Varshney, 2000) are playing important role in crop improvement (Budak *et al*., 2004).

[1] Centre for Plant Molecular Biology, TNAU, Coimbatore-641003 India.

SSR detects the allelic variation by way of repeat numbers with in a locus and one pair of SSR primer deals with one locus (Weising *et al*., 1992) where as RAPD detects both allelic and locus variations in a single assay and deals with insertions, deletions, point mutations which influence the base sequence of primer binding sites, allowing polymorphism to be detected (Williams *et al*., 1990). ISSR uses microsatellite, usually 16-25 bp long as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes (Zietkiewicz *et al*., 1994).

Various molecular marker techniques such as Restriction Fragment Length Polymorphism (RFLP) (Lebrun *et al.,* 1998), RAPD (Everard, 1999; Upadhyay *et al.,* 2004; Manimekalai and Nagarajan, 2006a), AFLP (Perera *et al.,* 1998), ISTR (Rohde *et al.,* 1995), SSR (Perera *et al.,* 1999; Meerow *et al.,* 2003) and ISSR (Manimekalai and Nagarajan, 2006b) have been used for analysis of coconut germplasm in different regions. Though comparison of molecular markers for their efficiency, effectiveness and informativeness in germplasm analysis has been carried out in many other crops (Katsiotis *et al.,* 2003 in pepper; Souframanien and Gopalakrishna, 2004 in blackgram; Archak *et al.,* 2003 in cashew; Prakash *et al.*, 2005 in coffee), such studies are limited only to a single comparison between AFLP and SSR in coconut (Teulat *et al.,* 2000). Therefore, in the present investigation, three marker systems *viz.,* RAPD, ISSR and SSR were compared for their informativeness and effectiveness in coconut germplasm analysis.

MATERIALS AND METHODS

Leaf samples

Leaf samples were collected from thirty-three germplasm accessions (one palm per accession) obtained from International Coconut Gene Bank for South Asia, Central Plantation Crops Research Institute (Research Centre), Kidu, Karnataka, India. The germplasm accessions consisted of 29 tall accessions, two dwarf accessions and two intermediate accessions. Out of 33 accessions, eight belong to South East Asia, nine accessions belong to South Pacific, four accessions belong to Atlantic and America, eleven accessions belong to South Asia and one accession belongs to Africa. DNA was extracted from 2 g of fresh leaf material from 33 coconut accessions using Nucleon phyto pure Plant DNA extraction kit (Amersham Bioscience).

RAPD analysis

RAPD analysis was carried out as described in Manimekalai and Nagarajan (2006a). Ten random primers were used to amplify the DNA. The PCR products were subjected to electrophoresis through a 1.2% agarose gel using 1X TBE buffer at 90 volts for 2 h in Bio-Rad submarine electrophoresis unit. The ethidium bromide stained gels were documented using the Alpha Imager^{TM} 1200 - Documentation and Analysis system (Alpha Innotech Corporation, USA).

ISSR analysis

ISSR analysis was carried out as described in Manimekalai and Nagarajan (2006b). Ten primers targeting the microsatellite regions were used to amplify the DNA. Primers were purchased from University of British Columbia, Canada). The PCR products were

subjected to electrophoresis through a 1.8% agarose gel using 1X TBE buffer at 90 volts for 2 h in Bio-Rad submarine electrophoresis unit (Bio-Rad Laboratories, USA). The ethidium bromide stained gels were documented using the Alpha Imager^{TM} 1200. (Alpha Innotech Corporation, USA and purchased through JH Bio Innovations Pvt. Ltd., Bangalore, India).

SSR analysis

SSR analysis was carried out as described in Perera *et al.* (2000). Primer sequences were obtained from CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement), Montpellier, France. Initially, Gradient PCR was used to standardize the annealing temperature for 10 primer pairs used in this study. The PCR products were separated in 3% Agarose gel (Agarose 1000 ^{TM}, Invitrogen).

Data analysis

Data were scored as 1 for the presence and 0 for the absence of a DNA band of each accession. Only the clear, unambiguous and reproducible bands were considered for scoring. The average Polymorphism Information Content (PIC) and Marker Index (MI) were calculated by applying the formulas given by Powell *et al.* (1996) and Smith *et al.* (1997).

The binary data matrices were entered into the NTSYS PC package (Exeter Software, New York). The data were analyzed using Qualitative routine to generate Jaccard's similarity coefficient.

Matrix comparison

The similarity matrix based on RAPD, ISSR and SSR markers were compared by the MXCOMP routine of NTSYS pc. The normalized Mantel statistic Z (Mantel, 1967) was used to determine the level of association between the matrices. Cophenetic correlation between the original similarity matrix and cophenetic matrix was calculated.

RESULTS

Comparison between RAPD, ISSR and SSR markers based on level polymorphism

Comparative account of RAPD, ISSR and SSR analysis is given in Table 1. Among the three marker types, ISSR markers produced the highest number of markers (117). RAPD (10 primers), ISSR (10 primers) and SSR (10 primer pairs) techniques produced 86, 97 and 92 polymorphic markers, respectively in 33 coconut germplasm accessions worldwide. The number of polymorphic markers, produced was 86, 97 and 92 for RAPD, ISSR and SSR, respectively. The average number of polymorphic markers produced per primer or primer pairs was 8.6, 9.7 and 9.2 for RAPD, ISSR and SSR, respectively. Among the three marker types, RAPD and ISSR produced 76.7 and 82.9% polymorphic markers, respectively. In contrast, SSR markers exhibited 100% polymorphism. Marker index and PIC were calculated for RAPD, ISSR and SSR markers. Mean PIC value was highest for SSR (0.78). Between ISSR and RAPD, the mean PIC was higher in ISSR (0.31) compared to RAPD markers (0.23). The extent of polymorphism

observed among the coconut germplasm by RAPD, ISSR and SSR analysis, respectively are shown in Figure 1.

Table 1. Level of polymorphism detected by RAPD, ISSR and SSR markers with coconut germplasm accessions.

Parameters	RAPD	ISSR	SSR
Number of primers	10	10	10
Total number of markers	112	117	92
Range of markers across primers	7-15	9-17	2-14
Number of polymorphic markers	86	97	92
Average number of polymorphic markers per primer	8.60	9.70	9.20
Percent polymorphism	76.70	82.90	100.00
Mean Polymorphism Information Content	0.23	0.31	0.78
Average Marker Index	2.10	3.00	7.60

Comparison between RAPD, ISSR and SSR markers based on similarity coefficient

A comparative account of RAPD and ISSR markers based on similarity range and Jaccard's similarity coefficient among coconut accessions is given in Table 2. SSR markers exhibited wide range of similarity (0.000-0.857) when compared to RAPD and ISSR markers. The average pair wise similarity based on Jaccard's coefficient was the lowest for SSR markers (0.215) and the highest for RAPD markers (0.710).

Table 2. Comparison of RAPD, ISSR and SSR markers based on similarity range and average similarity.

Parameters	RAPD	ISSR	SSR
Similarity range	0.531-0.871	0.431-0.853	0.000-0.857
Average pair wise similarity	0.69	0.62	0.21

Note: Similarity range = similarity is based on Jaccard's coefficient and Similarity range is based on maximum and minimum value in the similarity matrix. Average pair wise similarity = average of 528 numbers of comparisons in 33 x 33 similarity matrix.

Similarity matrices based on RAPD and SSR markers were compared using Mantel's test (Mantel, 1967) for matrix correspondence. There was high correlation between RAPD and ISSR matrices ('r' value = 0.510). A correlation value (r) greater than 0.5 will be statistically significant at 0.01 probability level if the number of taxonomic units exceeds 15 (Lapointe and Legendre, 1992). The correlation between RAPD and SSR matrices ('r' = 0.073)

and ISSR and SSR ('r' = 0.178) was less. Cophenetic correlation (r) for RAPD markers was 0.719, for ISSR markers was 0.734 and for SSR marker was 0.740.

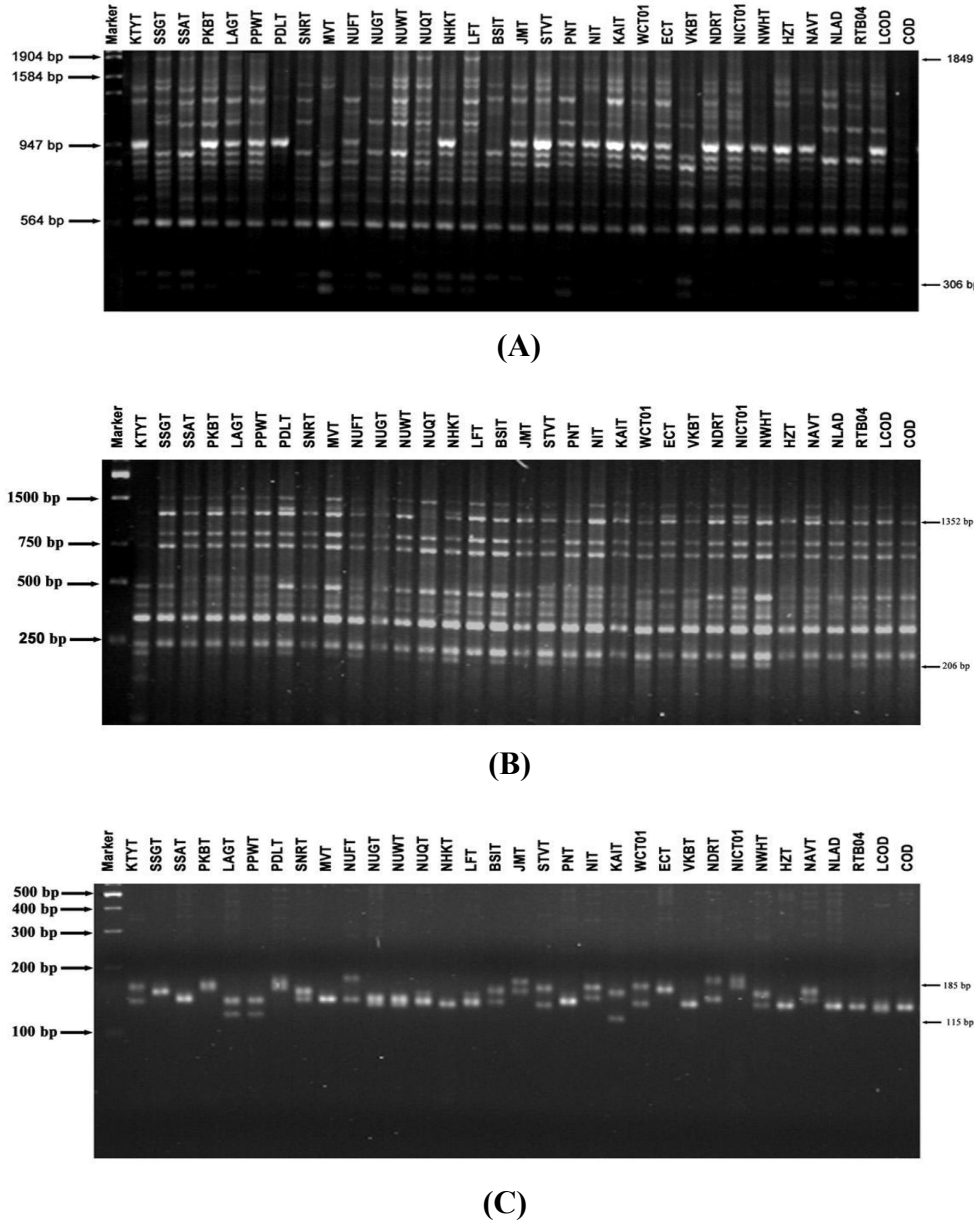

Fig .1. RAPD (A), ISSR (B) and SSR (C) profiles of Coconut accessions using OPC 13, VBC 834 and Cn Cir E2, primers respectably.

DISCUSSIONS

Though the molecular markers represent a sample of a plant genome, yet they are used to infer relationships of the entire genome among a set of populations. The distribution of loci detected by individual marker analysis methods will affect the precision of the resulting estimates of genetic distance (Nei, 1987). Hence, for the estimation of genetic diversity it is ideal that the loci detected are randomly dispersed throughout and must be a sample to represent the whole genome. The comparison of distribution of different individual markers needs genetic maps, but this is very time consuming (Lu *et al.,* 1996). An alternative approach has been proposed to estimate the relationships among the germplasm accessions derived from several marker techniques by using statistics like Mantel's test. Based on the Mantel's test, high correspondence between RAPD and ISSR (r = 0.51) similarity matrices and no correspondence between RAPD - SSR and ISSR - SSR similarity matrices was observed. No correlation between RAPD and SSR was reported in rice (Ravi *et al.,* 2003). The majority of the reports where correlation between molecular marker techniques has been found to exist have either been an autogamous crops or inbred lines, where genotypes tend to be homozygous (Lefebvre *et al.,* 2001; Virk *et al.,* 2000; Goulao *et al.,* 2001). Coconut, a cross-pollinated perennial species, is expected to have high levels of heterozygosity and also genetic backgrounds of the accessions are too diverse, characteristics correspondence among different molecular techniques may not show up.

The PIC provides an estimate of the discriminating power of a marker. The PIC value for ISSR markers was higher (0.31) than RAPD markers (0.23). Hence, the ISSR markers were more discriminative than RAPD markers. SSR markers showed a mean PIC value of 0.78 hence the best marker. As RAPD and ISSR markers are biallelic in nature, they can have maximum of 0.50 PIC value. But SSR markers are multi-allelic and their PIC values will be ranging from 0 if it is monomorphic to 1 if it is highly discriminative. Hence, it is evident that SSR markers are most discriminative among three marker systems namely RAPD, ISSR and SSR used in the study. The PIC values of 10 SSR primer pairs ranged from 0.4995 to 0.8872. It is in accordance with the report of Teulat *et al.* (2000) who reported the PIC values of SSR primers in the range of 0.470 to 0.900.

The mean similarity coefficient of 0.710, 0.620 and 0.215 was obtained based on RAPD, ISSR and SSR markers, respectively. Jaccard's similarity coefficient based on ISSR markers displayed a normal distribution, RAPD was skewed towards higher similarity, whereas, those based on SSR was skewed towards lower similarity. This again confirmed the better discrimination power and highly polymorphic nature of SSR markers. This superiority of SSR based on polymorphism detection has been reported earlier by Teulat *et al.* (2000), Dasanayake *et al.* (2003) and Perera *et al.* (2003) in coconut.

As it was noted that there was a good correspondence exists between RAPD and ISSR similarity matrices, ISSR may be used as an alternative to replace RAPD in the genetic diversity assessment. So that in future, ISSR markers would be the better tools than RAPD for genetic studies in coconut. In other crops also, it was concluded that ISSR were more informative than RAPD (Ajibade *et al.*, 2000; Galvan *et al.*, 2003). In apple, Goulao and Oliveira (2001) reported superiority of ISSR markers over RAPD and AFLP. The higher PIC values of ISSR primers (0.31) compared to RAPD (0.23) also added strength to the above statement.

By comparing all the three marker systems, between the two multilocus markers *viz.,* RAPD and ISSR, the latter was more efficient based on PIC value, percentage of polymorphism and better reproducibility. However for cultivar identification purpose SSRs are best tools, since they provide a higher level of polymorphism and reproducibility.

CONCLUSIONS

The present study compares the markers analysis among the heterogeneous coconut accessions. Low correlation between the marker types cautions the dependence on single marker technique in heterozygous crops like coconut. Results of the study suggest ISSR technique as an alternative marker system for RAPD for the genetic analysis in coconut. Both ISSR and SSR may be combined in the assessment of coconut genetic diversity among the germplasm and development of core collection to optimize the germplasm management for use in coconut breeding and conservation.

REFERENCES

Ajibade, S.R., Weeden, N.F. and Michite, S. (2000). ISSR analysis of genetic relationships in the genus *Vigna*. Euphytica. 111: 47-55.

Archak, S., Gaikwad, A.B., Gautam, D., Rao, E.V.V.B., Swamy, K.R.M. and Karihaloo, J.L. (2003). Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. Genome. 46: 362-369.

Budak, H., Bölek, Y., Dokuyucu, T. and Akkaya, A. (2004). Potential uses of molecular markers in crop improvement. KSU J. Sci. and Engi. 7: 75-79.

Dasanayake, P.N., Everard, J.M.D.T., Karunanayake, E.H. and Nandadasa, H.G. (2003). Characterization of coconut by microsatellite markers. Trop. Agric. Res. 15: 51-60.

Everard, J.M.D.T. (1999). An investigation towards developing a molecular approach to improve the efficiency of coconut breeding by RAPD marker assisted selection. CORD. 15: 115-130.

FAOSTAT (2004). Agriculture data. http://apps./fao.org.

Galvan, M.Z., Bornet, B., Balatti, P.A. and Branchard, M. (2003). Inter simple sequence repeat (ISSR) marker as a tool for the assessment of both genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris* L.). Euphytica. 132: 297-301.

Goulao, L. and Oliveira, C.M. (2001). Molecular characterization of cultivars of apple (*Malus x domestica* Borkh.) using microsatellie (SSR and ISSR) markers. Euphytica. 122: 81-89.

Goulao, L., Cabrita, L., Oliveira, C.M. and Leitao, J.M. (2001). Comparing RAPD and AFLP analysis in discrimination and estimation of genetic similarities among apple (*Malus domestica* Borkh) cultivars. Euphytica. 119: 259-270.

Gupta, P.K. and Varshney, R.K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica. 113**:** 163-185.

Katsiotis, A., Hagidimitriou, M., Drossou, A., Pontikis, C. and Loukas, M. (2003). Genetic relationship among species and cultivars of *Pistacia* using RAPDs and AFLPs. Euphytica 132: 279-286.

Lapointe, F.J. and Legendre, P. (1992). Statistical significance of the matrix correlation coefficient for comparing independent phylogenetic trees. Systematic Biology 41: 378-384.

Lebrun, P., N'Cho, Y.P., Seguin, M., Grivet, L. and Baudouin, L. (1998). Genetic diversity in coconut (*Cocos nucifera* L.) revealed by restriction fragment length polymorphism (RFLP) markers. Euphytica. 101: 103-108.

Lefebvre, V., Goffinet, B., Chauvet, J.C., Caromel, B., Signoret, P., Brand, R. and Palloix, A. (2001). Evaluation of genetic distances between pepper inbred lines for cultivar protection purposes: comparison of AFLP, RAPD and phenotypic data. Theoreti. Appl. Genet. 101: 741-750.

Lu, J., Knox, M.R., Ambrose, M.J., Brown, J.K.M. and Ellis, T.H.N. (1996). Comparative analysis of genetic diversity in pea assessed by RFLP and PCR based methods. Theoreti. Appl. Genet. 93: 1103-1111.

Manimekalai, R. and Nagarajan, P. (2006a). Interrelationship among coconut (*Cocos nucifera* L) germplasm accessions using RAPD technique. Genet. Resour. Crop Evol. DOI: 10.1007/s10722 - 005 - 1303z (online first).

Manimekalai, R. and Nagarajan, P. (2006b). Assessing genetic relationships among coconut (*Cocos nucifera* L.) accessions using Inter Simple Sequence Repeat Markers. Scientia Hort. 108: 49-56.

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209-220.

Meerow, A.W., Wisser, R.J., Brown, J.S., Kuhn, D.N., Schnell, R.J. and Broschat, T.K. (2003). Analysis of genetic diversity and population structure within Florida coconut (*Cocos nucifera* L.) using microsatellite DNA, with special emphasis on the Fiji Dwarf cultivar. Theoreti. Appl. Genet. 106: 715-726.

Nei, M. (1987). Molecular Evolutionary Genetics, Columbia University Press, New York.

Perera, L., Russell, J.R., Provan, J. and Powell, W. (1999) Identification and characterization of microsatellites in coconut (*Cocos nucifera* L.) and the analysis of coconut population in Sri Lanka. Mol. Ecol. 8: 344-346.

Perera, L., Russell, J.R., Provan, J. and Powell, W. (2000). Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (*Cocos nucifera* L.). Genome. 43: 15-21.

Perera, L., Russell, J.R., Provan, J. and Powell, W. (2003). Studying genetic relationships among coconut varieties/populations using microsatellite markers. Euphytica. 132: 121-128.

Perera, L., Russell, J.R., Provan, J., McNicol, J.W. and Powell, W. (1998). Evaluating genetic relationships between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. Theoreti. Appl. Genet. 96: 545-550.

Powell, W., Morgane, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S.V. and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP and SSR markers for germplasm analysis**.** Mol. Breed. 2: 225-238.

Prakash, N.S., Combes, M.C., Dussert, S., Naveen, S. and Lashermes, P. (2005). Analysis of genetic diversity in Indian robusta coffee genepool (*Coffea canephora*) in comparison with a representative core collection using SSR and AFLPs. Genet. Resour. Crop. Evol. 52: 333-343.

Ravi, M., Geethanjali, S., Sammeyafarheen, F. and Maheswaran, M. (2003). Molecular marker based genetic diversity analysis in rice (*Oryza sativa* L.) using RAPD and SSR markers. Euphytica. 133: 243-253.

Rohde, W., Kullaya, A., Rodriguez, J. and Ritter, E. (1995). Genome analysis of *Cocos nucifera* L. by PCR amplification of spacer sequences separating a subset of copia-like *Eco*RI repetitive elements. J. Genet. Breed. 49: 179-186.

Smith, J.S.C., Chin, E.C.L., Shu, H., Smith, O.S., Wall, S.J., Senior, M.L., Mitchell, S.E., Kresovich, S. and Ziegle, J. (1997). An evaluation of SSR loci as molecular markers in maize (*Zea mays* L.): Comparison with data from RFLPs and pedigree. Theoreti. Appl. Genet. 95: 163-173.

Souframanien, J. and Gopalakrisha, T. (2004). A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theoreti. Appl. Genet. 109: 1687-1693.

Tanksley, S.D., Young, N.D., Paterson A.H. and Bonierbale, M.W. (1989). RFLP mapping in plant breeding: New tools for an old science. Biotechnology. 7: 257-264.

Teulat, B., Aldam, C., Trehin, R., Lebrun, P., Barker, J.H.A., Arnold, G.M., Karp, A, Baudouin, L. and Rognon, F. (2000). An analysis of genetic diversity in coconut (*Cocos nucifera*) population from across the geographical range using sequence tagged microsatellites (SSRs) and AFLPs. Theoreti. Appl. Genet. 100: 764-771.

Upadhyay, A., Jayadev, K., Manimekalai, R. and Parthasarathy, V.A. (2004) Genetic relationship and diversity in Indian coconut accessions based on RAPD markers. Scientia Hort. 99: 353-362.

Virk, P., Zhu, S.J., Bryan, G.J., Jackson, M.T. and Ford-Lloyd, B.V. (2000). Effectiveness of different classes of markers for classifying and revealing variation in rice (*Oryza sativa*) germplasm. Euphytica. 112: 275-284.

Weising, K., Kaemmer, D., Weigand, F., Epplen, J.T. and Kahl, G. (1992). Oligonucleotide fingerprinting reveals various probe dependent levels of informativeness in chickpea (*Cicer arietinum*). Genome. 35**:** 436-442.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: 6531-6535.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. Genome. 20: 176-183.