Proceedings of the Second Annual Forestry Symposium 1996: Management and Sustainable Utilization of Forest Resources, Sri Lanka, 6-7 December 1996. (Eds. Amarasekera, H S, Ranasinghe, D M S H K and Finlayson, W). Published by Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Sri Lanka (1998)

USE OF RAPDs FOR ESTIMATION OF GENETIC DISTANCES BETWEEN POPULATIONS OF THE COCONUT PALM, *Cocos nucifera*, L.

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Abstract

Accurate estimation of genetic distances between and within species is vital in the conservation of heterogeneous tree populations, such as coconuts. Coconut germplasm is being conserved at present by the selection and preservation of ecotypes and in biased collections that are based on economic traits; such selections are likely to misrepresent the natural variability of the palm. To help in improving conservation methods, a DNA fingerprinting system, RAPDs (Random Amplified Polymorphic DNAs) is proposed.

RAPDs were investigated in 19 coconut types planted at the Coconut Research Institute, Sri Lanka, by using random primers of 10-12 nucleotides. Extracts of DNA from the leaves of four individual trees were pooled to represent each heterozygotic coconut type. Thirty-eight primers were tested in the RAPD-PCR and polymorphic bands and scored for presence or absence in each type. Pair-wise genetic distances were calculated according to Nei and Li's coefficient, and cluster analysis was performed on the basis of the resultant distance matrix.*

Eighteen primers detected a total 91 polymorphic bands across the coconut types screened. All these RAPDs were clear and reproducible over repeated runs. Grouping of 19 coconut types based on these RAPDs matched the currently accepted taxonomic grouping almost perfectly. This good match indicates that the RAPDs method is applicable as a sound genetic marker for the characterization of coconut genetic resources. The technique is simple and rapid. It can be developed without previous information about the DNA of the target plant. The cost is affordable for developing countries. The technique is now widely applied in the breeding and conservation of heterozygous perennial crops and forest trees.

Introduction

The significance of genetic diversity is often discussed with reference to the sustainable agriculture of staple food species, because breeders rely on a supply of genes from related

* PCR = single-primed polymerase chain reaction

wild species to develop new characteristics, such as resistance to pests and diseases (Pearce & Moran, 1995). Major collections of important crop plants are held in international centres around the world, which have been set up as repositories of the biodiversity available for each species. These collections are a valuable source of useful genes for plant breeders. Workers at many of the centres face problems in the efficient conservation, maintenance and exploitation of germplasm, because of the lack of true genetic information about the accessions that they hold.

Coconut play a prominent role in Sri Lankan culture. The annual per capita consumption is estimated to be 110 nuts. They provide food, drink, timber, fibre, copra, oil, and dessicated coconut. Since Sri Lanka is an island, the genetic diversity of its coconuts is obviously limited. Genetic erosion in coconut takes place at a rate of about 1% a year (Liyanage, 1985) and the National Replanting (NRP) envisages an annual planting rate of 2% to keep up a constant production rate. However, the way in which seed is supplied for the NRP, from seed gardens and seed palms in selected populations, aggravates genetic erosion.

Systematic collection and conservation of coconut germplasm was started in 1984 at the Coconut Research Institute (CRI) with a bias that favoured collections from drought-prone areas. Later, more of the agro-ecological zones were included. The CRI now holds 39 accessions in *ex-situ* gene banks (Perera et al., 1996). Conservation of the coconut germplasm in *ex-situ* gene banks is costly, and to increase the number of accessions demands very large areas of land and great expense for their establishment. Obviously all of the coconut populations cannot be conserved, and so it is important to choose a limited number of populations that give the best possible representation of the variability of the species. The development of methods for the identification of duplicates and for making core collections (representative samples of the whole collection) is of paramount importance.

At present the basis on which coconut germplasm is characterized is its vegetative and productive characters. The determination of most of these phenotypic traits is complicated, and they are sensitive to environmental differences, so that clear and detailed assessments of genetic diversity between these collections cannot easily be made. Most productive traits are more or less subject to both artificial and natural selection, and as a consequence their diversity may tend to reflect the action of selective forces rather than the genetic relationship between populations under study.

Information that is less biased can be obtained by the use of characters that are more directly related to the genome. The common DNA-based markers, Restriction Fragment Length Polymorphisms (RFLPs) and Random Amplified Polymorphic DNAs (RAPDs), have the advantage that they sample the genome directly. For crops that have not previously benefited from the use of molecular markers, the use of RAPDs has the greater advantage, because it does not require DNA-sequence information.

There have been few studies on coconut DNA markers, although there have been many on DNA probes used for the detection of mycoplasma-like organisms associated with diseases (Randles et al., 1992; Harrison et al., 1992; Rohde et al., 1993). The first reported work on the development of molecular markers in the coconut palm was the detection of a family of

highly repetitive *Eco*RI fragments of the genomic DNA, which showed a remarkable sequence homology to the copia-like group of retroposons (Rohde et al., 1992); this study was extended for the assessment of genetic diversity by constructing coconut-specific primers complementary to the repeats, for use in the PCR (Rohdé et al., 1995).

In the present study we have demonstrated the use of RAPDs to obtain diagnostic information for distinguishing coconut cultivars and sub-populations in Sri Lanka.

Materials and methods

The materials analysed by using RAPDs are listed in Table 1. DNA was extracted by the method of Dellaporte et al. (1988), with some modifications as described in Everard et al. (1996). Young leaves were picked from four individuals per accession, mixed, and used for DNA extraction. This procedure was thought to be necessary because of the great heterogeneity expected from *typica* cultivars.

Bulk DNA samples from each cultivar and each sub-population were used in PCR to generate random amplified DNA profiles. Initially thirty-eight oligonucleotide primers were used, 20 from the Operon Kit B (10-mer) supplied by OPERON technologies, and 18 (12-mer) from Dr C. Beard of the University of New England.

Table 1 : Coconut cultivars and sub-populations from CRI experimental stations at Pottukulama and Ambakelle, Sri Lanka, analysed by the use of RAPDs

Cultivar	Sub-population	Given notation
typica typica		T_1
typica typica		T_2
typica typica		T_3
typica typica	Moorcock	Mk
typica typica	Pitiyakande	Pk
typica typica	Goyambokka	Gb
typica typica	Akuressa	Ak
typica typica	Deberayaya	Db
typica ran thembili		RT
typica gon thembili		GT
typica pora pol		PP
typica san ramon		SR
aurantiaca King Coconut		KC
nana pumila (green dwarf)		DG
nana regia (red dwarf)		DR
nana ebernia (yellow dwarf)		DY
Nana russet (brown dwarf)		DB
Cameroon red dwarf		CRD
Brazilian green dwarf		BGD

The PCR procedure was based on that of Williams et al. (1991). The amplification reactions were performed in a thermocycler (Bartlet Instruments) in a volume of 25 ml containing 1 × *Taq* reaction buffer, 2 mM magnesium chloride, 100 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 0.2 mM of a single 10-mer primer, 50-100 ng of genomic DNA, and 0.5 units of *Taq* DNA polymerase (Bresatec). The reaction was allowed to progress in 45 repeating cycles of three phases, viz: denaturing of the genomic DNA (1 minute at 94°C), annealing of primers (1 minute at 36°) and extension of primers (2 minutes at 72°). The amplification products were made visible by 1.5% agarose gel electrophoresis.

After a series of PCRs the primers that gave strong consistent banding patterns over three repeated runs were selected and used in the final RAPD-PCR analysis. The sizes of the bands generated by each primer were estimated by the size standards of HindIII DNA. Specific amplification products were scored as present (1) or absence (0) for each DNA sample. An index of genetic similarity (F) and pair-wise distances (1 - F) were calculated by the matching coefficient method of Nei and Li (1979):

$$F = 2N_{AB1}/(N_A + N_B)$$

In this equation N_{AB1} = the number of bands present in both samples A and B
N_A = the number of bands present in sample A
N_B = the number of bands present in sample B

The data matrix of genetic distances was obtained by using the software package, RAPDISTANCE (version 1.02) developed by John Armstrong, Adrian Gibbs, Rod Peakall and Geroge Weiler of the Australia National University The package also creates the Neighbour Joining Tree, to analyse unweighted pair groups.

Results

Of 38 primers tested, 18 effectively primed the amplification of coconut genomic DNA, and 17 revealed polymorphisms. Plate 1 shows an example of a gel used for scoring RAPDs. Table 2 shows the sequences of the 38 primers and their performances. The 17 primers produced a total of 160 fragments, ranging from 200 bp to 2200 bp in size, 91 (56.8%) of which were polymorphic. The mean number of amplification products per primer is 9.4 ± 0.6 and the mean number of polymorphic bands per primer is 5.4 ± 0.7. TheG + C content of the OPB primers was designed to be more than 60% and in 12-mer primers they varied between 42% and 75%. The failure of certain primers to generate RAPD profiles of the reduced number of fragments produced by some of them cannot strictly be attributed to the G + C content of the primer in this assay, because some of the primers, even with G + C contents as high 70-75% failed to generate good DNA profiles. It is more likely therefore that the combination of primer sequence and DNA template is more decisive than G + C content in yielding a good RAPD-PCR profile.

Table 2 : Sequences and codes of random primers and the number of monomorphic and polymorphic fragments amplified in 19 coconut accessions

Code number	Primer sequence (5'-3')	G + C (%)	Number of amplified fragments	Number of polymorphic fragments	Range of molecular weights
12mer1	CTCCGGGGTGTG	75			
12mer2	TCATCCCGCTTC	58	9	1	200-1800
12mer3	AACCCTTTTCAA	33			
12mer4	TGGCCGCCCGTT	75			
12mer5	TGGCCACCGTTG	66			
12mer6	TAGCCGCCGTTG	66	6	5	300-1800
Check1	TCATAAGGCGAT	42			
Check2	AATCGCTTATGA	33	10	7	550-1800
Check3	GGGACTGGAAA	50			
Check4	TTTTCCCAGTCC	50			
Check5	AGGTCATCAGAC	50			
Check6	GTCTGAATGACC	50	8	7	300-1600
Check7	GGCAACGCGGTC	75	13	13	300-2000
Check8	ACCGCGATTGCC	66	8	1	200-1100
Check9	AAGCTTCACTCC	50			
Check10	GGGAGTAAGCTT	50			
Check11	GACATACGCGAA	50			
Check12	TCGCCGTATGGC	75			
OPB1	GTTTCGCTCC	60	12	11	200-1000
OPB2	TGATCCCTGG	60			
OPB3	CATCCCCCTG	60			
OPB4	GGACTGGAGT	60	10	6	250-1800
OPB5	TGCGCCCTTC	70	9	3	250-1800
OPB6	TGCTCTGCCC	70	4	2	800-1600
OPB7	GGTGACGCAG	70	10	4	300-1800
OPB8	GTCCACACGG	70	8	4	300-1600
OPB9	TGGGGGACTC	70			
OPB10	CTGCTGGGAC	70			
OPB11	GTAGACCCGT	60	7	5	300-2000
OPB12	CCTTGACGCA	60	14	6	200-1800
OPB13	TTCCCCCGCT	70	8	5	650-2000
OPB14	TCCGCTCTGG	70			
OPB15	GGAGGGTGTT	60	8	5	350-1200
OPB16	TTTGCCCGGA	60			
OPB17	AGGGAACGAG	60	10	5	300-2000
OPB18	CCACAGCAGT	60	12	9	200-2200
OPB19	ACCCCCGAAG	70			
OPB20	GGACCCTTAC	60			

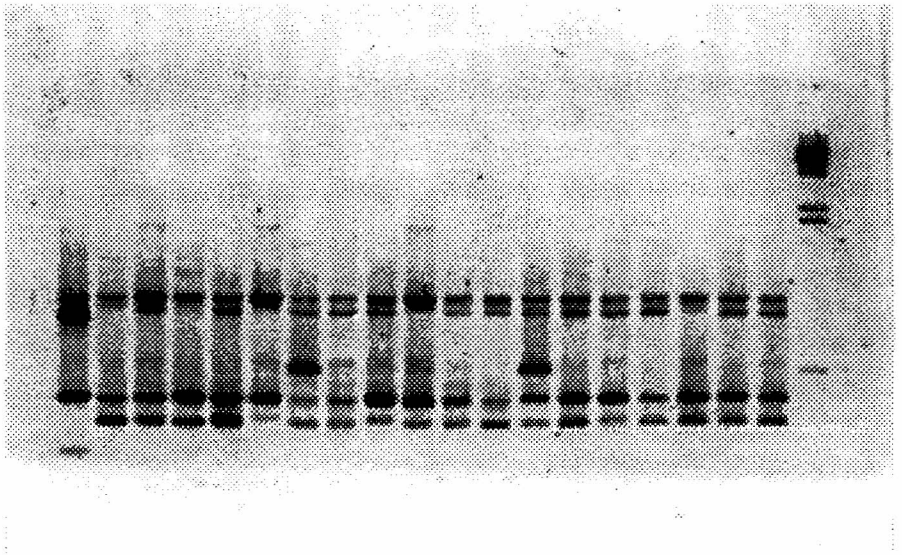

Plate 1:. Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer OPB1. Lanes 1, 2 and 3 *typica typica* commercial tall (replicates 1, 2 and 3); lane 4 *typica* gon thembili; lane 5 *typica* pora pol; lane 6 *typica* ran thembili; lane 7 *typica typica* Moorcock; lane 8 *typica typica* Pitiyakanda; lane 9 *typica typica* Goyambokka; lane 10 *typica typica* Akuressa; lane 11 *typica typica* Deberayaya; lane 12 *typica San ramon;* lane 13 *aurantiaca* king coconut; lane 14 dwarf green; lane 15 dwarf yellow; lane 16 dwarf red; lane 17 dwarf brown; lane 18 Brazilian green dwarf; lane 19 Cameroon red dwarf; lane 20 HindIII. Digested lambda DNA is the standard DNA marker (the sizes of bands 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end, in kb, are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56).

The pair-wise genetic distance matrix based on Nei and Li's coefficient included all 19 accessions. The genetic distances in the matrix were used to find mean distances between and within cultivars (Table 3). The average distance between *typica* and *nana* accessions is clearly more than the average genetic distances within either group, indicating a clear separation of the two groups. The genetic distance between *typica* and *san ramon* is higher than the genetic distance between *nana* and *san ramon*. This is an unexpected result, because taxonomically *san ramon* has hitherto been placed as a *typica* cultivar. The genetic distances indicate that the intermediate *aurantiaca* type shares a greater proportion of genomic DNA with *nana* than it does with *typica;* this result agrees with the morphological similarities between *aurantiaca* and *nana*.

The Neighbour Joining Tree of the 19 accessions is shown in Fig. 1. In general, the dendrogram reflects the separation of the accessions based on our understanding of their morphological characters and reproductive behaviour. The hierarchical distribution of the

accessions in the tree indicates a clear separation of *typica* from *nana*. Clustering within *typica*, at lower hierarchical levels, grouped together 3 individuals of *typica typica* (the commercial type) as expected. The separation of other *typica typica* sub-populations from *typica typica* commercial type is an indication of the selection pressure on the commercial type. At the lowest level the sub-populations clustered in accordance with their geographical locations. Moorock and Pitiyakanda originally came from the wet intermediate zone, and Deberayaya, Akuressa, and Goyambokka from the dry intermediate zone. The separation of the morphologically distinct *typica* cultivars ran thembili, pora pol, and gon thembili at a much higher level is also much as expected.

Table 3 : Mean genetic distances between and within cultivars of the coconut palm as revealed by RAPDs

Sources of diversity	Mean genetic distance	Standard deviation	Range
Within all the 19 accessions	0.33	0.11	0.10-0.52
Within *typica*	0.24	0.06	0.12-0.52
Within *nana*	0.19	0.05	0.10-0.37
Between *typica* and *nana*	0.41	0.04	0.32-0.29
Between *typica* and *aurantiaca*	0.43	0.05	0.34-0.52
Between *typica* and *San ramon*	0.44	0.03	0.27-0.36
Between *nana* and *aurantiaca*	0.32	0.03	0.29-0.37
Between *nana* and *San ramon*	0.29	0.03	0.27-0.36
Between *aurantiaca* and *San ramon*	0.49		

Clustering of *nana* cultivars is in even closer agreement with distinctions based on morphological features. The classic, more dwarf-like cultivars (short stature without a root bole) – dwarf red, green and brown, Cameroon red and Brazilian green – grouped well within the main cluster, separating from more tall-like cultivars *san ramon*, king coconut and dwarf yellow. Sri Lankan dwarf yellow shows great morphological variation within the cultivar and is often misclassified as gon thembili, a tall cultivar.

The grouping of coconut accessions at all levels, based on RAPDs, reflected the best fits that could be expected in terms of shared morphology, reproductive behaviour, and geographical isolation. This good general agreement with the traditional classification strongly supports the applicability of RAPDs for the detection of genetic diversity in the coconut palm.

Discussion

The RAPD technique has demonstrated its ability to discriminate plant populations at various taxonomic levels; for example genera of the *Lolium/Fetusa* complex (Stammer et al., 1995), species of *Hordeum* (Gonzalez & Ferer, 1993), genotypes in mustard (Jain et al., 1994), accessions of oil palm (Shah et al., 1994), cultivars of apple (Koller et al., 1994) and clones of cocoa (Wilde et al., 1992). The RAPDs method is claimed to be the most accurate one for

investigating relationships among populations within a species (Gonzalez & Ferer, 1993), and this claim is borne out by this present study.

The genetic diversity seen in heterogeneous populations complicates the analysis of genetic variance that is due to their inherent within-population variation. The estimation of genetic variation by the RAPDs analysis of bulk genomic DNA samples appears to be a sound solution. It has proved successful with heterogeneous populations of alfalfa (Kangfu & Pauls, 1993), celery (Yang & Quiros, 1993) and wild rice (Virk et al., 1995).

The optimum number of primers required for the assessment of genetic diversity depends on many factors. Obviously, if the assessment is between more distant groups, more polymorphism per primer can be expected. However, by pre-selection of primers for the DNA substrate, a precise assessment of genetic diversity can be achieved with a very small number of primers. For instance Tinker et al. (1993) distinguished 27 inbred lines of barley with seven primers. A considerable amount of genetic polymorphism has been detected within the coconut species. The genetic distance of 0.524 between the most distant accessions, and an average of 5.3 polymorphic fragments per primer, indicate the presence of substantial genetic diversity.

Clustering of coconut accessions by RAPDs reflects the currently accepted morphological classification (Liyanage, 1958), including recent additions to the taxonomy (Perera et al., 1996). The overall genetic similarity within the dwarf cluster is high compared with what it is in the tall cluster; this result was to be expected, because of the predominantly outbreeding behaviour of *typica*, and also because the dwarf types have been selected and maintained in cultivation (Benabadis, 1992).

The clustering of the Philippine tall type *san ramon* with dwarfs was unexpected, but is a very exciting result because of the markedly different characters possessed by *san ramon* compared with the Sri Lanka tall types. *San ramon* palms are very tall and erect, with stout trunks and massive root boles. Their nuts are very large and round, with kernels almost as twice as thick as those of the Sri Lankan commercial tall type. Even more interestingly, this result is in conformity with Carpio's (1982) studies on Philippine coconut populations, based on the electrophoresis of pollen proteins.

However, because of the dominant inheritance of heterozygous perennial crops, the usefulness of RAPDs in generating linkage maps for them is limited. The lack of inbred lines, back-cross populations, or anther-culture dihaploids in the coconut makes the problem even worse. This consideration points to the immediate need for developing F_2 populations from the existing F_1 ones. There is still a possibility for tagging simple genetic traits by the application of bulk segregant analysis, as suggested by Michelmore et al. (1991). This method analyses two bulked DNA samples gathered from individuals that are segregated by RAPDs in a single population. Each bulk is composed of individuals that differ for a specific phenotype or of individuals at either extreme of a segregating population. There is therefore a good chance of tagging characters such as resistance to diseases.

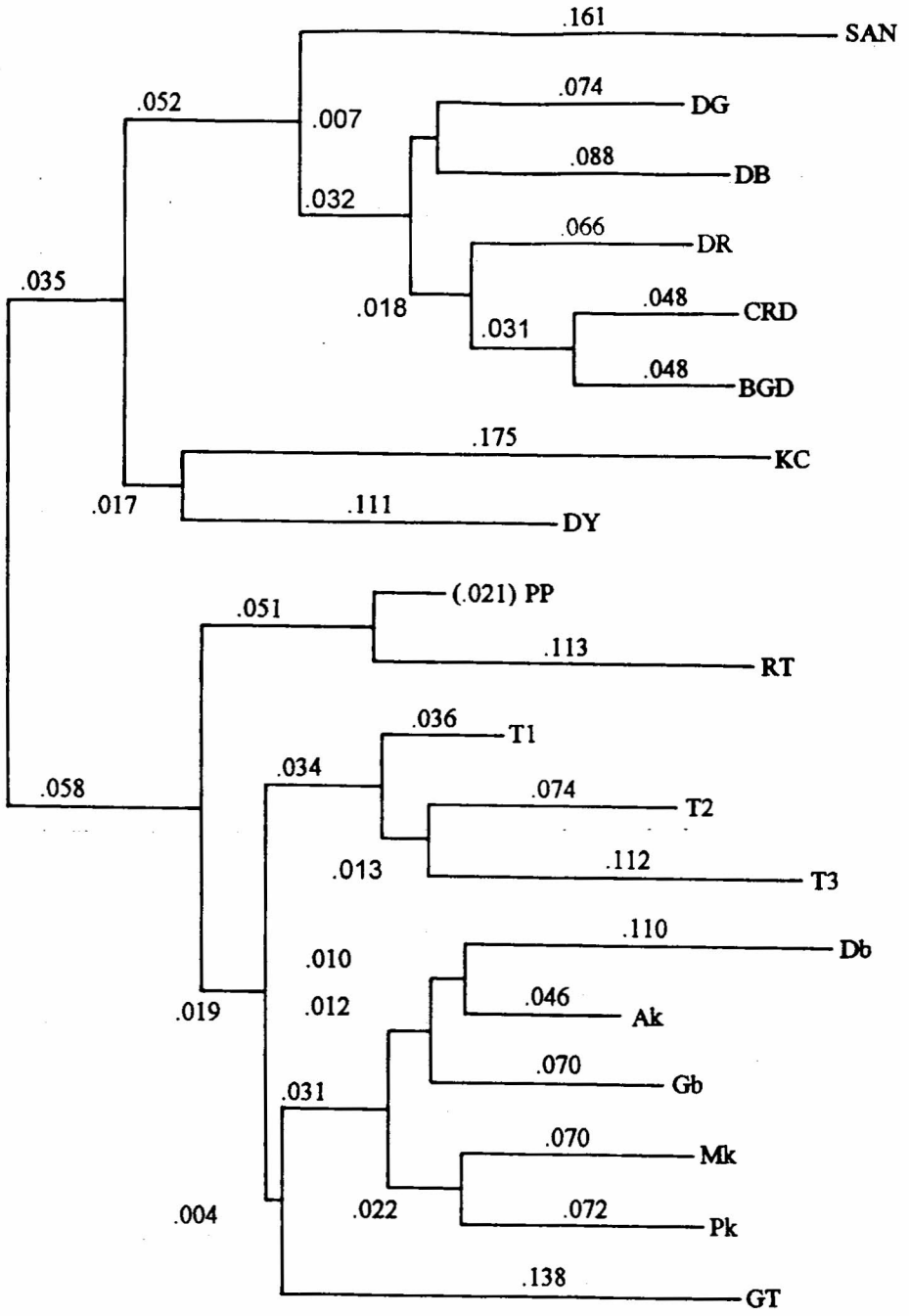

Figure 1 : Dendrogram of coconut accessions determined by clustering pair-wise genetic distances estimated by Nei and Li's coefficient

Acknowledgements

In general, the findings of this study provide clear evidence of the potential of RAPDs as useful molecular markers in improving breeding and conservation strategies for the coconut palm. The speed, low cost, efficiency, and reliability of the RAPD methodology, in conjunction with numerical analysis, make it an excellent tool for more effective management of gene banks, in regard to duplicate identification, estimation of diversity, and monitoring of genetic erosion, and for making better use of the germplasm collections in the identification of core populations.

The authors are grateful to Dr Keith Gregg, Molecular Biologist, University of New England, for providing laboratory facilities for this study, and for his valuable suggestions. The work was funded by the Agricultural Research Project of the Ministry of Agriculture, Sri Lanka.

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