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Evaluating genetic relationships between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling

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Abstract PCR-based DNA profiling of coconut palms indigenous to Sri Lanka was conducted using amplified fragment length polymorphism (AFLPs). A total of 322 amplification products were generated from the 42 genotypes with eight pairs of primers (*Eco*RI and *Mse*I). Overall most variation was detected in the tall (Typica) rather than the intermediate (Aurantiaca) and dwarf (Nana) forms. A hierarchical analysis of molecular variance (AMOVA) was used to quantify and partition levels of variability into between- and within-form components. This revealed that for the inbreeding dwarf and intermediate forms most variation was observed between, rather than within, forms. In contrast, the outbreeding tall forms exhibited as much variation within as between forms. These observations have important implications for the maintenance and collection of coconut germplasm. This study also provided insights into the genetic (as opposed to phenotypic) relatedness of coconut accessions. Morphologically the Aurantiaca group of accessions are considered to be intermediate between the tall and dwarf accessions. Estimation of genetic relatedness based on AFLP analysis identified the Aurantiaca group as being more similar to the dwarf rather than the tall group. In addition, putative duplicate accessions were identified in the Aurantiaca group. Information emerging from this study will facilitate the management of coconut germplasm and optimise the choice of genetically divergent parents for crossing.

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Introduction

The coconut palm (*Cocos nucifera* L.) is a major plantation crop in the tropics and occupies an important position in the international vegetable oil market. Coconut is also the most widely grown plantation crop occupying 21% of the total land under agriculture in Sri Lanka (Fernando et al. 1995) and is one of the three major plantation crops on the island. In addition to its commercial value as an oil crop for generating foreign exchange, coconut also plays a major role in the Sri Lankan diet and social life.

C. nucifera ($2n = 2x = 32$) is a member of the monocotyledonous family Arecaceae (Palmaceae) and is the only species of the genus. There are conflicting theories regarding the origin and domestication of coconut (Whitehead 1976; Harries 1978), and the varieties and forms of coconut have been described by different workers (Narayana and John 1949; Gangolly et al. 1957; Liyanage 1958; Menon and Pandalai 1958) based on morphology and breeding behaviour. The existence of varieties of coconut in Sri Lanka has been recognised since the last century (Seeman 1856; Trimen 1898) but a systematic classification of coconut in Sri Lanka was first conducted in 1958 (Liyanage 1958) and identified three varieties; Typica, Nana and Aurantiaca, Aurantiaca being intermediate between Typica and Nana. Presently the locally available gene pool (the indigenous cultivars) has been classified into three varieties and 15 forms based on their breeding habit and morphological characters such as stature, the colour of nuts, and quantitative and qualitative differences in fruit components (Liyanage 1958; Wickramaratna and Rathnasiri 1986; Perera et al. 1992).

For a successful coconut breeding programme, accurate estimates of genetic diversity and its partitioning within and between gene pools are important considerations. This is particularly important for coconut due to its long generation time and the associated costs of maintaining mature palms. The assembly of elite breeding populations and their thorough evaluation is therefore a priority. Morphological traits have the disadvantages of being influenced by both environmental and genetical factors and may therefore not provide an accurate measure of genetic diversity. At present, however, the evaluation and characterisation of coconut germplasm is based exclusively on morphological and reproductive traits (Fernando et al. 1995). Attempts to characterise coconut populations based on isozymic, polyphenol and carotenoid differences have been reported in many countries with different coconut populations (Benoit 1979; Jayasekera 1979; Carpio 1982; Jay et al. 1989; Fernando 1995). With the availability of molecular-marker techniques, the characterisation of genetic diversity in coconut at the DNA level has also been reported (Rohde et al. 1992; Ashburner and Rohde 1994; Rohde et al. 1995; Everard 1996).

A new high-multiplex PCR-based method for DNA profiling [amplified fragment length polymorphism (AFLP)] has recently been described by Zabeau and Vos (1993) and Vos et al. (1995). This assay has the potential to generate a large number of polymorphic genetic loci (Vogel et al. 1994; Powell et al. 1996) and has been used to analyse genetic diversity in rice (Mackill et al. 1996), lettuce (Hill et al. 1996), soybean (Maughan et al. 1996), tea (Paul et al. 1997), barley (Ellis et al. 1997; Pakniyat et al. 1997), and an endangered plant, *Astragalus creminophylax* (Travis et al. 1996). In the present study we have used AFLPs to investigate genetic relatedness between accessions of coconut maintained at the Coconut Research Institute of Sri Lanka. The primary motive for this study was to establish the extent of genetic diversity between palms in order to facilitate the planning of coconut breeding programmes based on an optimal choice of parents for crossing.

Materials and methods

Plant material

Plant materials were obtained from the *ex-situ* coconut gene bank of the Coconut Research Institute of Sri Lanka and consisted of 42 genotypes from eight forms of the variety Typica (tall type), four forms of the variety Nana (dwarf type), and two forms of the variety Aurantiaca (intermediate type). Each form was represented by three individuals. For the variety Typica the following forms were studied; typica, nawasi, gon-thembili, ran-thembili, pora-pol, bodiri, kamandala and san ramon (Fernando 1987), an introduced form from the Philippines. For the variety Nana the following forms were studied; dwarf green, dwarf yellow, dwarf red and dwarf brown. The variety Aurantiaca was represented by the forms king coconut and rathran-thembili. With the exception of san ramon all the other forms studied were indigenous to Sri Lanka.

Table 1 Sequences of primers and adaptors used

*Eco*RI		5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGATGGTTAA-5′
*Mse*I		5′-GACGTGAGGTCCTGSG-3′ 3′-TACTCAGGACTCAT-5′
*Eco*RI + 0 primers	E00	5′-GACTGCGTACCAATTC-3′
*Eco*RI + 3 primers	E34	5′-GACTGCGTACCAATTCAAT-3′
	E43	5′-GACTGCGTACCAATTCATA-3′
	E52	5′-GACTGCGTACCAATTCCCC-3′
	E73	5′-GACTGCGTACCAATTCGGG-3′
	E88	5′-GACTGCGTACCAATTCTGC-3′
*Eco*RI + 4 primers	E181	5′-GACTGCGTACCAATTCCCCC-3′
*Mse*I + 0 primers	M00	5′-GATGAGTCCTGAGTAA-3′
*Mse*I + 3 primers	M56	5′-GATGAGTCCTGAGTAACGC-3′
	M66	5′-GATGAGTCCTGAGTAAGAT-3′
*Mse*I + 4 primers	M140	5′-GATGAGTCCTGAGTAAAGTC-3′
	M307	5′-GATGAGTCCTGAGTAATCAG-3′
	M238	5′-GATGAGTCCTGAGTAAGATC-3′

DNA extraction

DNA was isolated from frozen fresh young coconut leaves by a modified version of the miniprep protocol for the isolation of total DNA developed by Dellaporte et al. (1983), essentially as described by Everard (1996). DNA was purified by phenol/chloroform premixed with isoamyl alcohol. The yield of DNA was determined by comparison against standards and was approximately 0.01 mg/1 g of leaf material.

Amplified fragment length polymorphism

The AFLP procedure followed that described by Vos et al. (1995). Approximately 0.5 μg of DNA was digested with *Eco*RI and *Mse*I. The adaptors, non-selective primers, selective primer pairs and their sequences are listed in Table 1. In each case only *Eco*RI primers were radioactively labelled using [γ^{33}P]. ATP as described by Zabeau and Vos (1993). Eight primer combinations were employed to detect AFLP polymorphism between the 42 genotypes. The amplification products produced with the eight different primer combinations were separated on 6% polyacrylamide gels for 1.45 h at 80 W and then transferred to chromatography paper and dried on a gel drier for 2 h at 80°C. Gels were exposed to Kodak X-Omat 100 film for about 5–6 days.

Statistical analysis

AFLP products were scored as present (1) or absent (0) on autoradiographs to create a binary matrix. Diversity values based on phenotype frequency were calculated for individual primer pairs using Nei's (1978) index:

$$\hat{H} = 1 - \sum [p_i^2],$$

where p_i is the frequency of the i^{th} phenotype, phenotype in this case being defined as the AFLP profile observed for a specific genotype using a single primer pair. A matrix of inter-phenotypic distances based on combined data from all AFLP primer pairs was constructed using the shared-band similarity measure of Nei and Li (1979).

Principal co-ordinate and cluster analyses based on the similarity matrix were performed with GENSTAT (1987) V5.31 using group average linkage to produce a 3-D plot and a dendrogram showing the relationships between the accessions studied. The similarity matrix was also used to perform a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) essentially as described by Huff et al. (1993) using the ARLEQUIN software (V1.0). The number of permutations for significance testing was set at 1000 for all analyses.

Results

Profiling of palms

The eight pairs of primers generated a total of 322 scoreable products among the 42 genotypes studied, of which 198 bands (61.4%) were polymorphic. Part of a typical gel is shown in Fig. 1. The information generated for each primer pair is given in Table 2 and shows that primer pairs differ in their discriminating power but that overall genetic diversity indices exceed 0.9. Based on all 322 products, 38 of the 42 individuals could be uniquely genotyped. Two individuals within each of the king coconut and rathran-thembili forms could not be distinguished and have identical AFLP profiles.

AMOVA analysis

The nested AMOVA was used to partition total genetic diversity between varieties, between forms within varieties, and within forms. There were significant differences ($P < 0.001$) detected for all hierarchies within the AMOVA analysis but most variation was found between varieties (47%) (Table 3). This analysis was repeated for each variety group (Typica, Nana and Aurantiaca) independently and revealed differences in the pattern of variability detected. For the Typica (tall) group of forms, the genetic variability was distributed approximately equally between and within forms. In contrast, for both Nana (dwarfs) and Aurantiaca (intermediates) forms more genetic variation was detected between rather than within forms, although formally the partitioning of variation was non-significant ($P = 0.099$) in the Aurantiaca group.

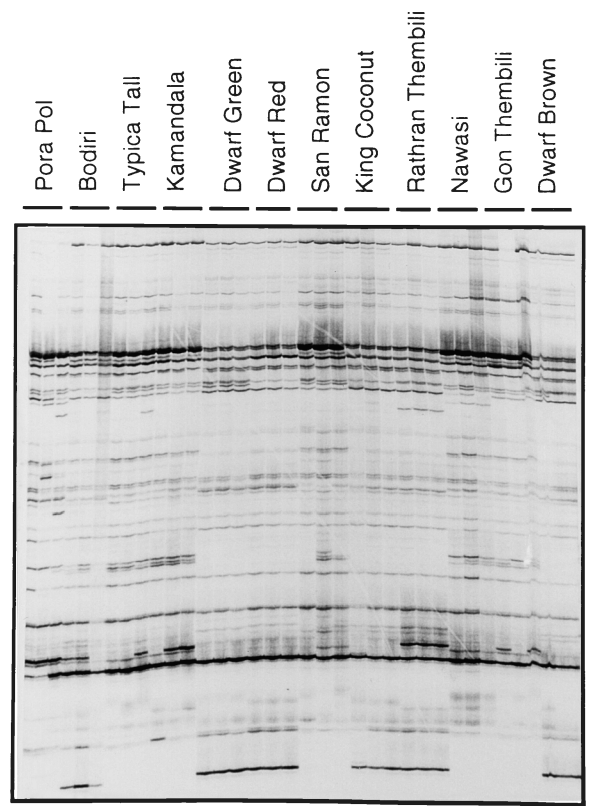

Fig. 1 AFLP profiles generated using primer combination E73/M56

Table 2 Number of polymorphic bands produced and the level of phenotypic diversity detected using Nei's index

Primer combination	Total no. of bands	No. of polymorphic bands detected				% Polymorphic bands	Phenotypic diversity				No. of unique genotypes identified
		Tall	Dwarf/ Aurantiaca	Shared	Total		Tall	Dwarf	Aurantiaca	Total	
E52/M307	38	27	22	22	27	71	0.950	0.899	0.279	0.950	27
E73/M140	28	22	18	16	24	86	0.882	0.780	0.612	0.916	12
E73/M56	64	32	38	30	40	62	0.956	0.865	0.720	0.970	33
E43/M238	36	18	19	15	22	61	0.938	0.860	0.500	0.956	21
E181/M140	56	30	30	25	35	62	0.954	0.893	0.500	0.960	26
E88/M66	33	13	13	11	15	45	0.903	0.720	0.500	0.925	7
E34/M140	40	21	20	20	21	52	0.952	0.776	0.450	0.957	23
E73/M307	27	13	14	13	14	52	0.880	0.780	0.500	0.928	18
Total	322	176	174	138	198						
Average						61	0.918	0.821	0.590	0.946	

Table 3 Analysis of molecular variance (AMOVA) for coconut genotypes analysed with AFLPs

Source of variation	*df*	MSD	Variance component	% Total variance	Probability
Between varieties	2	57.94	4.02	47.0	< 0.001
Between forms within varieties	11	9.75	2.61	30.6	< 0.001
Within forms	28	1.92	1.92	22.4	< 0.001
Between Typica (tall) forms	7	10.00	2.53	48.5	< 0.001
Within Typica forms	16	2.38	2.38	52.3	
Between Nana (dwarf) forms	3	10.16	2.82	62.3	< 0.001
Within Nana forms	8	1.70	1.70	37.7	
Between Aurantiaca forms	1	7.17	2.20	81.6	= 0.099
Within Aurantiaca forms	4	0.50	0.50	18.4	

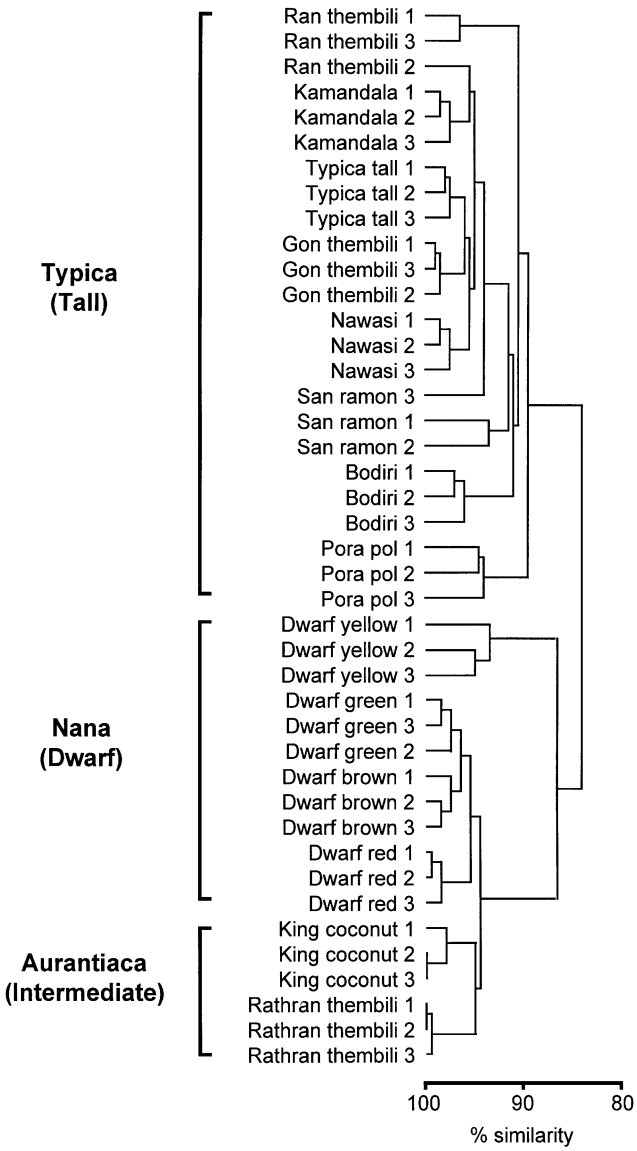

Fig. 2 Dendrogram of coconut (*C. nucifera* L.) genotypes derived by average linkage cluster analysis

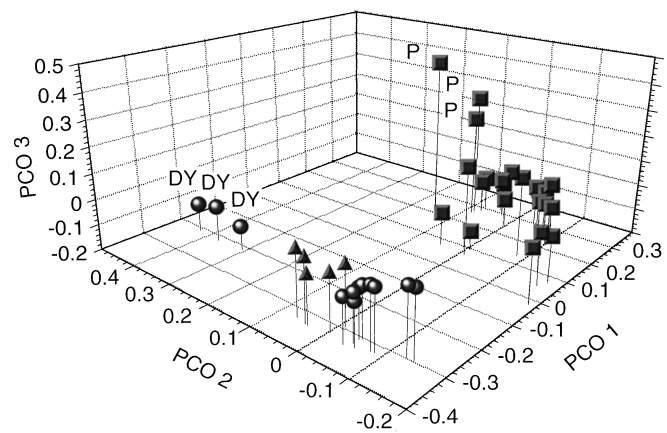

Fig. 3 Principal co-ordinate plots of 42 genotypes of coconut (*C. nucifera* L.). ■ – tall genotypes, ● – dwarf genotypes, ▲ – Aurantiaca genotypes, DY – dwarf yellow, P – pora-pol

Principal co-ordinate and cluster analysis

A dendrogram based on group average linkage cluster analysis of the pair-wise genetic distances for the 42 genotypes is shown in Fig. 2. The resultant dendrogram divided the 42 coconut genotypes into two main groups, one represented by all the Typica (tall type) and the other comprising two subgroups corresponding to the Nana (dwarf type) and Aurantiaca (intermediate type) genotypes. This grouping is generally consistent with the existing morphological classification of indigenous coconut varieties and forms.

The first three principal co-ordinate axes account for 40.7, 9.6 and 6% of the total variation respectively and identify four main groupings (Fig. 3). The first axis clearly discriminates the tall genotypes from the remaining genotypes. The second PCO discriminates the dwarf yellow genotypes from the remaining Nana and Aurantiaca genotypes. The pora-pol genotypes are differentiated from the other genotypes by the third PCO axis.

Discussion

This study was undertaken to evaluate the extent and range of genetic diversity available in Sri Lankan coconut germplasm. Based on AFLP analysis, the genetic base of coconut germplasm in Sri Lanka would appear to be narrow with estimates of genetic similarity exceeding 81%. Total genetic diversity was partitioned between different groups of accessions using an analysis of molecular variance (AMOVA). This methodology, originally described by Excoffier et al. (1992) and extended by Stewart and Excoffier (1996), has been used to examine diversity in buffalo grass (Huff et al. 1993), *Pinus strobus* (Hamelin et al. 1995), *Eucalyptus globulus* (Nesbitt et al. 1995), *Astragalus cremnophylax* (Travis et al. 1996), and *Iliamna corei* (Stewart et al. 1996). The pattern of variability detected in coconut was related to the breeding structure of the palms with the tall, outbreeding coconuts (variety Typica) exhibiting higher overall levels of diversity than inbreeding dwarfs (variety Nana) and Aurantiaca coconuts. This trend is also apparent in the pattern of variability, with the inbreeding varieties exhibiting more variation between rather than within forms. Quantification of the level of variability in the different varieties indicates that for the dwarf and Aurantiaca varieties more genetic variation will be captured by sampling different forms rather than collecting more individuals within forms.

The genetic relationships determined by AFLPs are generally consistent with previous studies (Everard 1996). For example the Aurantiaca group of genotypes, although considered as intermediate for certain morphological characters, are genetically (based on AFLPs) more similar to the dwarf group of genotypes than the Typica group. This observation is in agreement with the RAPD variation described for some genotypes from the same Sri Lankan germplasm (Everard 1996). With the exception of san ramon, all other genotypes evaluated are indigenous to Sri Lanka. Hence it is surprising that this introduced variety did not occupy a more genetically distant position in the dendrogram. More importantly, it illustrates the need to obtain genetic estimates of diversity rather than exclusively relying on morphological and site-of-origin data for germplasm analysis . Further evidence of the value of AFLPs to discriminate between genotypes is provided by the position of the dwarf yellow forms relative to the other genotypes (Figs. 2, 3). Morphological and agronomical data generated by Manthriratna (1972) indicates that the dwarf yellow form is more vigorous and productive than the other Nana forms and this is reflected in the position of this form relative to the other accessions studied.

One of the main attractions of the AFLP method is its high multiplex ratio (Powell et al. 1996; Rafalski et al. 1996) which means that a large number of amplification products are generated in a single reaction. Furthermore, the method is generic and does not depend on the availability of sequence information. It is therefore particularly appropriate for poorly characterised germplasm such as coconut, providing a rapid route to genotypic rather than phenotypic evaluation. For example, two accessions within the king coconut and rathran-thembili forms were shown to be indistinguishable based on AFLP analysis (Fig. 2). It would therefore be reasonable to eliminate these putative duplicates from the collection.

In conclusion, the present study has demonstrated the power of AFLP technology to provide a high-resolution analysis of genetic diversity in coconut. Such objective measurements of genetic diversity can help define priorities, reduce the costs associated with the maintenance of elite coconut palms, and optimise the choice of parents for crossing.

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