Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (*Cocos nucifera* L.)

L. Perera, J.R. Russell, J. Provan, and W. Powell

Abstract: We have used eight pairs of simple sequence repeat (SSR) primers to analyse the genetic diversity in 130 individuals of coconut (*Cocos nucifera* L.) comprising 75 tall individuals and 55 dwarf individuals, representing 94 different coconut ecotypes throughout the world. A total of 51 alleles were detected, with an average of 6.4 alleles per locus. Fifty alleles were detected in tall coconuts (talls; mean alleles/locus 6.3) compared with only 26 (mean/locus 3.3) in dwarfs, and the average diversity value in talls (0.589) was also significantly higher than that in dwarfs (0.348). Using the eight SSRs we were able to uniquely discriminate 116 of the 130 individuals. A phenetic tree based on D_{AD} (absolute distance) values clustered individuals into five groups, each mainly composed of either talls or dwarfs. These results provide evidence in support of previous hypotheses concerning the dissemination of coconut, as well as important new information for conservation and breeding purposes.

Key words: coconut, *Cocos nucifera*, microsatellites, SSR, genetic diversity.

Résumé : Des paires d'amorces spécifiques à huit microsatellites (SSRs) ont été employées pour analyser la diversité génétique parmi 130 cocotiers (*Cocos nucifera* L., 75 de grande taille et 55 nains) représentant 94 écotypes différents provenant de partout dans le monde. Au total, 55 allèles ont été détectés pour une moyenne de 6,4 allèles par locus. Cinquante allèles ont été détectés parmi les cocotiers de grande taille (une moyenne de 6,3 allèles par locus) alors que seuls 26 allèles étaient présents chez les cocotiers nains (une moyenne de 3,3 allèles par locus). La valeur moyenne de diversité génétique chez les grands cocotiers (0,589) était supérieure à celle observée chez les cocotiers nains (0,348). À l'aide des huit microsatellites, il a été possible de distinguer 116 des 130 individus. Un arbre fondé sur les valeurs D_{AD} a groupé les individus en cinq groupes, chacun composé principalement de cocotiers grands ou nains. Ces résultats appuient les hypothèses concernant la dissémination du cocotier et apportent d'importantes informations nouvelles en vue de la conservation et de l'amélioration génétique du cocotier.

Mots clés : cocotier, *Cocos nucifera*, microsatellites, SSR, diversité génétique.

[Traduit par la Rédaction]

Introduction

Coconut 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. Currently, coconut is mainly an oil crop; rich in lauric acid, with a variety of other uses in addition to commercial oil production (Harries 1995). Coconut is a member of the monocotyledenous family Arecaceae (Palmaceae), subfamily Cocoideae and the monospecific genus *Cocos*. The existence of related genera of coconut in South America (Cook 1910; Purseglove 1985) and coconut's long history in the Eastern hemisphere has led to controversy over its centre of diversity. Fremond et al. (1966) summarised the main reasons for considering a Southeast Asian origin for coconut, and Melanesia is considered as the most likely region for coconut domestication along the coasts and islands between Southeast Asia and the Western Pacific (Harries 1995). According to Whitehead (1976), coconut spread both west and east from this putative centre of diversity. Purseglove (1985) suggested an alternative route for the evolution of coconut from a South American ancestor that could have been disseminated by ocean currents from South America to Polynesia.

Coconut has been distributed to many different parts of the world including Central and South America, East and West Africa, Southeast Asia, East Asia and the Pacific islands. Dissemination was achieved by floating in sea currents (Ohler 1984) and subsequent germination on the shore, followed by further human dispersal. Geographic isolation, introgressive hybridisation, mutation, and selection are the

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Table 1. Primer information, number of alleles detected, and diversity statistics for eight coconut microsatellite primers.

Locus	Repeat	Primers (5′–3′)	EMBL no.	Size range (bp)
CAC2	$(CA)_{12}(AG)_{14}$	AGCTTTTTCATTGCTGGAAT	AJ011865	210–254
		CCCCTCCAATACATTTTTCC	AJ011866	
CAC3	$(CA)_{13}$	GGCTCTCCAGCAGAGGCTTAC	AJ011867	187–203
		GGGACACCAGAAAAAGCC	AJ011868	
CAC4	$(CA)_{19}(AG)_{17}$	CCCCTATGCATCAAAACAAG	AJ011869	182–216
		CTCAGTGTCCGTCTTTGTCC	AJ011870	
CAC6	$(AG)_{14}(CA)_{9}$	TGTACATGTTTTTTGCCCAA	AJ011871	150–168
		CGATGTAGCTACCTTCCCC	AJ011872	
CAC8	$(AG)_{10}(CA)_{9}$	ATCACCCCAATACAAGGACA	AJ011873	188–210
		AATTCTATGGTCCACCCACA	AJ011874	
CAC10	$(TA)_{6}$CATA	GGAACCTCTTTTGGGTCATT	AJ011875	195–205
	$(CA)_{11}(TA)_{8}$	GATGGAAGGTGGTAATGCTG	AJ011876	
CAC11	Complex	GATCTTCGGCGTTCCTCA	AJ011877	156–170
	$(CA)_{n}(TA)_{n}$	TCTCCTCAACAATCTGAAGC	AJ011878	
CAC13	$(CA)_{9}(TA)_{5}$A	GGGTTTTTTAGATCTTCGGC	AJ011879	158–172
	$(TA)_{4}(CA)_{6}$	CTCAACAATCTGAAGCATCG	AJ011880	
				Mean
				Total

most likely causes of population differentiation of coconut. In addition, the mode of dispersal of coconut is likely to have resulted in founder effects influencing population differentiation (N'Cho et al. 1993). In the recent history of coconut, Europeans encouraged large-scale planting of coconut and participated in large-scale movements of germplasm which had previously been geographically isolated.

The primary classification of coconut is based on stature and breeding habit with two main categories: tall and dwarf (Menon and Pandalai 1958). Tall coconuts (talls) grow to a height of about 20–30 m and are allogamous, late flowering, and their nuts are medium to large in size. They are hardy and thrive in a wide range of environmental conditions. Dwarf coconuts grow to a height of about 10–15 m and are autogamous, early flowering, and generally produce a large number of small nuts with distinctive colour forms. Harries (1978) proposed two main types of talls: Nie kafa, which evolved naturally and was disseminated by ocean currents and Nie vai, which evolved as a result of selection from Nie kafa under cultivation and was disseminated by man. Introgression of these two types and further selection and dissemination by man produced the wide range of varieties and pan-tropical distribution of coconut seen today (Harries 1978). Despite the higher degree of autogamy, dwarfs cross-pollinate with one another and also with talls. The origin of the dwarf coconut is still inconclusive.

Assessment of the genetic diversity present within a species is a prerequisite for future sustainable breeding efforts. To date there are over 300 recorded ecotypes of coconut (Coconut Genetic Resources Network Database v. 2.2 COGENT/IPGRI), with evaluation and characterization being mainly carried out on morphological and reproductive traits (Fernando et al. 1995). Molecular markers provide an important technology for evaluating levels and patterns of genetic diversity and have been utilised in a variety of plant species (Powell et al. 1995, 1996; Rafalski et al. 1996). Among the various DNA marker methods currently available (Rafalski et al. 1996) that can be used to examine genetic diversity at the molecular level, the most informative polymorphic marker system to date is microsatellites, or SSRs (simple sequence repeats, Tautz and Renz 1984; Powell et al. 1996). Their high information content, co-dominance, and PCR-based detection mean that SSRs are an ideal tool for many genetic applications (Bruford and Wayne 1993; Queller et al. 1993; Dallas et al. 1995).

In this study we have utilised primers we previously designed to amplify polymorphic SSRs in coconut (Perera et al. 1999) to study the levels and patterns of genetic diversity and population genetic structure of coconut palm obtained from various parts of the globe.

Materials and methods

Plant material and DNA isolation

A total of 130 individuals comprising 75 talls and 55 dwarfs representing 94 different varieties of coconut from different coconut-growing regions were used in the study. All of the ecotypes were represented by a single individual, except for the Sri Lankan germplasm, with three individuals per ecotype. The details of the materials used are presented in Fig. 2 and further information can be obtained from the authors on request. DNA was isolated from frozen young coconut leaf material using a modification of the protocol described by Perera et al. (1998).

Construction of a small insert genomic library enriched for SSRs

SSRs were identified using a pre-cloning enrichment procedure described by White and Powell (1997). The DNA was enriched for $(CA)_n$ repeats using biotinylated oligomer $(CA)_{13}$ and the enriched fraction was then cloned into λ-ZAP phage vector (Statagene). Positive recombinant clones were identified by hybridising with an end-labelled $(CA)_{13}$ oligomer and were sequenced on an ABI377 automated sequencer (PE Biosystems, U.S.A.). Primers were de-

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Number of alleles			Genetic diversity ± s		
All	Tall	Dwarf	All	Tall	Dwarf
9	9	5	0.71±0.02	0.83±0.02	0.42±0.05
5	5	2	0.38±0.04	0.55±0.04	0.07±0.03
8	8	3	0.76±0.02	0.79±0.01	0.57±0.02
9	9	5	0.70±0.02	0.79±0.02	0.48±0.04
9	8	4	0.72±0.02	0.74±0.03	0.30±0.06
5	5	3	0.47±0.03	0.62±0.03	0.16±0.05
3	3	2	0.47±0.02	0.40±0.03	0.51±0.01
3	3	2	0.49±0.01	0.50±0.02	0.48±0.04
6.4	6.3	3.3	0.59±0.02	0.65±0.02	0.35±0.02
51	50	26			

signed using PRIMER (Whitehead Institute of Biomedical Research, v. 0.5, U.S.A.) and synthesised by Genosys (Paris, France). For a complete description of SSR characterization and primer design, see Perera et al. (1999).

Detection of microsatellite polymorphism

Eight microsatellites that previously showed clear polymorphisms (Perera et al. 1999) were pre-selected for use in this study (Table 1). The PCR conditions were as follows: denaturing at 94°C for 3 min, an annealing step at 65°C for 1 min followed by a seven-step touchdown decreasing by 1°C at each step to 58°C, and an extension step at 72°C for 2 min. Conditions for the last 27 cycles were 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. PCR was performed in a total volume of 20 mL containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 mM dNTPs, 10 pmol ^{32}P end-labelled forward primer, 10 pmol reverse primer, 0.1 U Taq polymerase (Boehringer Mannheim) and 20 ng genomic DNA. Reaction products were separated on 6% polyacrylamide gel in 1× TBE buffer and visualised by autoradiography.

Data analysis

Diversity values based on phenotype frequencies were calculated for each nuclear SSR locus using Nei's unbiased statistic, $\hat{H}$ (1987):

$$[1] \quad \hat{H} = \frac{n\left(1 - \Sigma\left[p_i^2\right]\right)}{n - 1}$$

where n = number of individuals analysed and p_i is the frequency of the ith allele. The sampling variance was calculated as:

$$[2] \quad V(\hat{H}) = 2 / \left[2(n-2)\left[\Sigma p_i^3 - (\Sigma p_i^2)^2\right] + \Sigma p_i^2 - (\Sigma p_i^2)^2\right]$$

Distances between individuals were calculated from SSR repeat sizes using the computer program MICROSAT v. 1.5 (E. Minch, Stanford University, U.S.A.) to calculate the absolute distance (D_{AD}) metric which is based on the sum of the number of repeat differences between genotypes and is similar to the $\hat{d}_{ij}$ metric of Goldstein et al. (1995):

$$[3] \quad \hat{d}_{ij} = \frac{\sum_{k=1}^{L} \left|a_{ik} - a_{jk}\right|}{L}$$

where a_{ik} and a_{jk} are the repeat sizes of the ith and jth individuals at the kth locus respectively and L is the number of loci analysed. A neighbour-joining tree showing relationships based on D_{AD} genetic distances was constructed using the NEIGHBOR and DRAWTREE options in the PHYLIP package (v. 3.57c, Joe Felsenstein, University of Washington, U.S.A.).

Results

Levels of polymorphism detected

Allelic diversity was measured in 130 coconut individuals. All loci were polymorphic, revealing single locus profiles. A total of 51 alleles were observed, ranging from 3 alleles for *CAC11* and *CAC13* to 9 alleles for *CAC2*, *CAC6*, and *CAC8*, with an average of 6.4 alleles per locus (Table 1). An example of allelic polymorphism is shown in Fig. 1. Diversity values ($\hat{H}$) ranged from 0.386 for *CAC3* to 0.762 for *CAC4*, with a mean gene diversity of 0.589. A total of 50 alleles were observed for talls, ranging from 3 alleles for *CAC11* and *CAC13* to 9 alleles for *CAC2* and *CAC6*, with an average of 6.3 alleles per locus. Only 26 alleles were observed for dwarfs, ranging from 2 for *CAC3*, *CAC11*, and *CAC13*, to 5 for *CAC2* and *CAC6*, with an average of 3.3 alleles per locus. The mean gene diversity of talls was 0.649, ranging from 0.398 for *CAC11* to 0.830 for *CAC2*. In contrast, the mean diversity index of dwarfs was much lower (0.348), and is comparable with the observed reduction in number alleles detected.

The allele frequencies for each locus and each group (tall and dwarf) are presented in Table 2. Twenty-four unique al-

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Table 2. Allele frequencies at eight SSR loci in coconut genotypes studied. Most common allele at each locus is shown in bold.

Locus	Allele (bp)	Frequency		
		Tall	Dwarf	All
CAC2	210	0.007	-	0.004
	220	0.007	-	0.004
	232	0.061	-	0.035
	234	0.142	-	0.083
	240	0.270	0.038	0.173
	246	0.061	0.094	0.075
	248	0.128	0.057	0.098
	252	0.034	0.057	0.043
	254	**0.291**	**0.755**	**0.484**
CAC3	187	0.027	-	0.015
	197	**0.623**	**0.963**	**0.768**
	199	0.226	0.037	0.146
	201	0.116	-	0.067
	203	0.007	-	0.004
CAC4	182	0.007	-	0.004
	186	**0.314**	**0.462**	**0.377**
	188	0.193	0.077	0.143
	200	0.036	-	0.020
	204	0.186	-	0.107
	208	0.193	-	0.111
	212	0.064	**0.462**	0.234
	216	0.007	-	0.004
CAC6	150	0.033	-	0.019
	152	0.155	-	0.088
	154	0.101	0.009	0.062
	156	0 020	-	0.012
	158	**0.304**	0.273	0.291
	160	0.277	**0.672**	**0.446**
	162	0.081	0.009	0.050
	164	0.014	0.036	0.023
	168	0.014	-	0.008
CAC8	188	0.050	0.020	0.040
	196	0.014	-	0.008
	198	0.130	**0.830**	**0.430**
	200	0.210	0.080	0.160
	202	-	0.020	0.008
	204	0.113	0.047	0.085
	206	0.010	-	0.010
	208	0.040	-	0.020
	210	**0.430**	-	0.250
CAC10	195	0.007	-	0.004
	197	0.188	-	0.107
	201	**0.542**	**0.917**	**0.702**
	203	0.243	0.074	0.171
	205	0.021	0.009	0.016
CAC11	156	0.270	**0.500**	0.368
	158	**0.723**	**0.500**	**0.628**
	170	0.007	-	0.004
CAC13	158	0.423	**0.833**	**0.594**
	162	**0.570**	0.167	0.402
	172	0.007	-	0.004

leles were detected for talls with only one for dwarfs. The frequency distribution of allele sizes was greater in talls compared to dwarfs. At 4 of the 8 loci, the most frequent allele is the same for both tall and dwarf coconuts with a loss of allelic richness being apparent in the dwarf palms sampled (e.g., *CAC2*, 254 bp; *CAC3*, 197 bp; *CAC6*, 160 bp; and *CAC10*, 210 bp). In contrast, at locus *CAC8*, the most frequent allele in the tall group is not found within the dwarf genotypes sampled. The 210-bp allele for tall and 198-bp allele for dwarf in Sri Lankan coconut populations appeared to be fixed at locus *CAC8*. Heterozygotes were evident in both talls and dwarfs but their number in dwarfs was negligible.

Genetic relatedness of accessions and discriminating power of microsatellites

The phenetic tree showing relationships between the individuals studied is shown in Fig. 2. It can be seen that there are two groups (I and II) and that the second of these is further divided into a number of subgroups (designated IIa–d; Table 3). Group I consists only of talls and includes all the Sri Lankan talls studied as well as four of the five African talls. Groups IIa and IIc consist largely of dwarfs (23 of 30 and 24 of 29 individuals, respectively), whilst groups IIb and IId are mostly made up of talls. The Sri Lankan intermediate (*Aurantiaca*) ecotype King Coconut and Rathran Thembili are grouped together with the dwarfs in group IIc. While SE Asian talls are found in all groups, almost half of them are assigned to group IIb (Table 3).

The eight microsatellites uniquely discriminated 116 of the 130 individuals evaluated. The 14 individuals that could not be uniquely genotyped fell into two groups; one represented by 10 individuals belonging to 4 dwarf ecotypes from Sri Lanka, and the other comprising 4 dwarf ecotypes from Thailand. All of the tall palms studied could be individually identified based on their DNA profile.

Discussion

This paper presents the use of microsatellite DNA markers to investigate the level of genetic diversity, distribution of genetic variation, and genetic relatedness in coconut genotypes. A key observation is the large number of alleles detected with coconut microsatellites providing a multi-allelic, co-dominant marker system. The level of discrimination is higher than that reported for isozymes (Fernando et al. 1995), RAPDs (random amplified polymorphic DNAs Everard 1996, Ashburner et al. 1997) and RFLPs (restriction fragment length polymorphisms, Lebrun et al. 1998), but lower that of reported for AFLPs (amplified fragment length polymorphisms) by Perera et al. (1998), who evaluated a subset of the materials described in the present study. However, assignment of allelic classes to related accessions is more difficult with AFLPs.

The overall low genetic diversity in dwarf coconuts when compared with the talls is related to their breeding habit; tall coconuts being predominantly out-breeding and dwarf coconuts being predominantly inbreeding (Liyanage 1949). The possibility of domestication of dwarf palms from a small number of tall palms also cannot be ruled out as a causative factor, giving rise to these low levels of genetic diversity in

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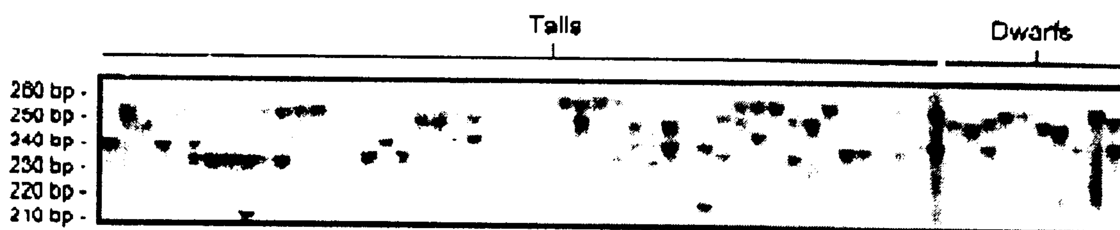
Table 3. Distribution of talls and dwarfs in phenetic tree classified by geographical origin.

	Tall					Dwarf					
Group	SE Asia	S Asia	America	Africa	Pacific	SE Asia	S Asia	America	Africa	Pacific	Total
I	4	24	1	4	1	-	-	-	-	-	34
IIa	5	-	-	-	2	15	5	1	1	1	30
IIb	17	-	1	-	3	5	-	-	-	-	26
IIc	2	1	-	1	-	12	12	-	1	-	29
IId	7	-	-	-	2	-	1	-	-	1	11
Total	35	25	2	5	8	32	18	1	2	2	

Fig. 1. Example of polymorphisms detected by primer CAC2.



dwarfs. The fact that almost all the alleles of the dwarfs are common to talls suggests that the dwarf population is a subset of the main tall population, and has evolved directly from tall coconut. The bimodal distribution of dwarf alleles at locus *CAC4* suggests that two separate domestication events may have taken place in the case of dwarf coconuts. The presence of both 212-bp and 186-bp alleles in Sri Lankan dwarfs and complete absence of these two alleles in Sri Lankan talls suggests that dwarf coconuts in Sri Lanka represent a separate introduction, rather than having evolved from Sri Lankan tall coconuts. This is further highlighted by the fact that no Sri Lankan dwarfs are found in the same group as Sri Lankan talls. This represents a similar finding to the RFLP work of Lebrun et al. (1998), who found that African dwarfs did not group with African talls. The fact that all the alleles found in the American and African samples are found in all the other regions (i.e., America and Africa display no unique alleles) suggests American and African coconuts evolved from a subset of the tall gene pool and may represent an introduction. The high genetic diversity of Philippines dwarfs (data not shown) suggests that the Philippines is the probable centre of domestication of dwarfs.

The results of the phenetic tree of the 130 individuals (Fig. 2) agrees with both Harries' (1978) theories on the natural and human-assisted dissemination of coconut and the RFLP study of Lebrun et al. (1998). It is accepted that the coconut palm has existed on the Atlantic coast of Africa and South America and around the Caribbean region for less than 500 years (Purseglove 1985) and that there is a great similarity between these coconuts and coconuts in East Africa, India, and Sri Lanka (Harries 1977). The grouping of Mozambique tall coconut, which Harries (1977) suggests as the main source of coconuts to East Africa and Atlantic Coast of America, with Cameroon Kiribi tall, West African tall and all Sri Lankan talls in the phenetic tree confirms the validity of Harries' theory of natural and human-assisted coconut germplasm dissemination in this region (Fig. 2 and Table 3). Interestingly, the Comoro tall (African) accession studied here fell out with the main group I and seemed to be intermediate between this group and the other coconuts studied, also noted by Lebrun et al. (1998). The grouping of South Asian dwarfs, American dwarfs and African dwarfs suggests that the dissemination of dwarf coconut has followed the same route as the tall coconuts. The clustering of Panama (Manarge) tall with Solomon Island tall from the Pacific region (Fig. 1, group IIb) and presence of both these accessions and two other Pacific talls in a branch of the phenetic tree containing Southeast Asian coconuts is in agreement with Whitehead's (1976) eastward movement of coconuts from the Southeast Asian to Pacific region, and subsequently from the Pacific region to the Pacific Coast of America (Fig. 2 and Table 3), although the position of Panama (Aguadulce) tall (in group I with the Sri Lankan talls) would appear to contradict this. Despite this, these results are largely in agreement with the results of ISTR (inverse sequence-tagged repeats) analysis (Rohde et al. 1995), which grouped Panama talls with Polynesian coconuts.

These SSR primers also represent a valuable resource for coconut breeders. With the use of multiplex PCR (simultaneous amplification of several genetic markers in a single reaction) coupled with the fluorescence-based DNA detection and semi-automated allele-sizing technology (Ziegle et al. 1992), it is now possible to generate accurate, large quantities of genetic information with microsatellite DNA markers (Mitchell et al. 1997). This semi-automated technology coupled with the microsatellites identified in this study will have an important role in the identification of coconut accessions that represent a core genepool for ex situ maintenance. This is an important objective given the recalcitrant nature of coconut seed. Furthermore, SSR-genotyping will allow the efficient selection of parents for coconut-breeding programmes, where the choice of genetically divergent parents will maximize heterosis, and thus increase hybrid vigour (Foale 1991).

In summary, we have demonstrated that SSR polymorphism provides a valuable tool for the analysis of coconut

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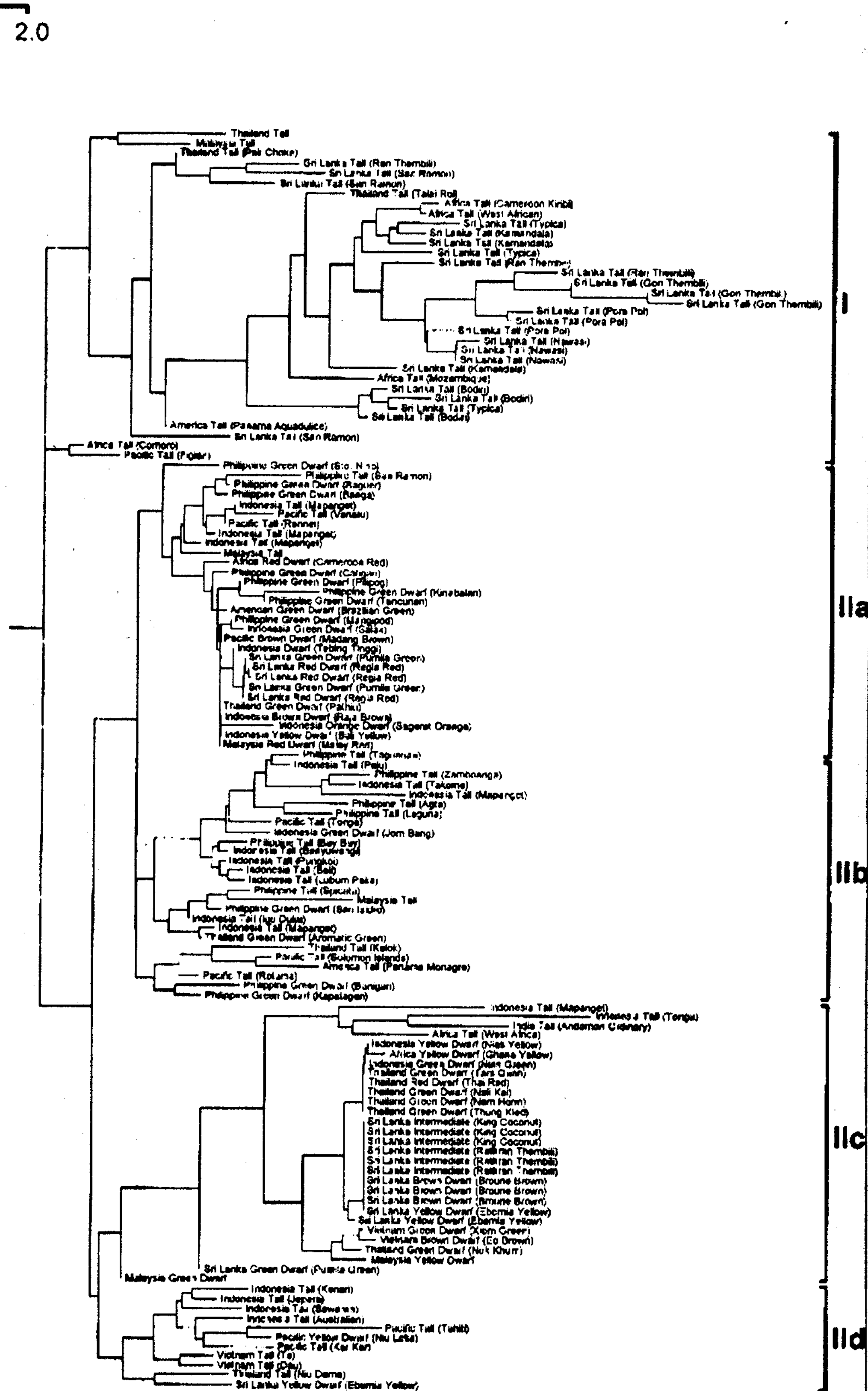
Fig. 2. Neighbour-joining phenetic tree based on D_{AD} genetic distances showing relationships between ecotypes studied.



populations. The patterns of variation detected confirm previous theories on the evolution of coconuts and their pathways of dissemination throughout the world, and are in agreement with recently published RFLP work on similar taxa. In addition, these results have implications for conservation purposes, since they can be used to identify a core genepool for ex situ conservation, as well as for future breeding programmes.

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