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Levels and distribution of genetic diversity of coconut (*Cocos nucifera* L., var. *Typica* form *typica*) from Sri Lanka assessed by microsatellite markers

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Summary

The coconut variety *Typica*, form *typica*, commonly known as Sri Lanka tall coconuts is the most widely exploited and grown variety in Sri Lanka. Under the coconut bio-diversity conservation programme, several *Typica* populations have been collected by island-wide surveys and planted *ex situ*. Thirty-three coconut populations were subjected to microsatellite assay with eight coconut-specific microsatellite primer pairs in order to study the levels and distribution of genetic variation of the collected materials for formulating future collection strategies and selecting parents for the breeding programme. A total of 56 alleles were detected ranging from 3 to 10 alleles per primer pair with an average of 7 alleles per locus. Overall a very high level of genetic diversity was detected (0.999) for all the populations studied ranging from 0.526 for population Debarayaya to 0.683 for population Dickwella. Only four introduced coconut populations, i.e. Clovis, Margeret, Dickwella, Mirishena and an embryo-cultured population were clearly separated from the resulting dendrogram. A very high level of within population variation (99%) accounted for native populations suggests a common history and a restricted genetic base for native Sri Lankan tall coconuts. Categorization of alleles into different classes according to their frequency and distribution confirmed the results of the dedrogram and concluded the adequacy of single large collection from the entire target area to represent the total genetic diversity in Sri Lanka. This study discusses useful information regarding conservation and breeding of coconut in Sri Lanka.

Introduction

The existence of coconut palms in Sri Lanka dates back to 101–77 B.C. (Sri Lanka census of agriculture in 1982, 1987). However, the growing of coconut in some organized form in Sri Lanka began around the 5th century A.D. (Karunanayake, 1982). The available coconut germplasm in Sri Lanka is categorized into three distinct varieties vz. *Typica*, *Nana* and *Aurantiaca* (Liyanage, 1958) and fifteen forms within varieties (Liyanage, 1958; Wickramaratne & Rathnasiri, 1986; Perera et al., 1992). The variety *Typica*, form *typica*, commonly known as Sri Lanka tall coconuts is the most widely exploited and grown variety in Sri Lanka. Coconut is the most widely grown plantation crop in Sri Lanka and it best grows at low altitudes near the coast under conditions of high humidity and temperature between 27–37 °C with moderately drained and well-aerated soil. However, coconut can grow in a range of environments and is found to be tolerant to number of abiotic stresses. In Sri Lanka, coconut can be found growing in range of environmental conditions other than the optimal conditions and maintain their productivity despite the stress conditions. In addition evidence suggests that the coconut plantations in Sri Lanka, in particular the large ones have undergone preferential selections for various characters such as yield, nut size, nut shape, nut colour, kernel thickness and tolerance to drought, pest and disease (Perera et al., 1996).

The genetic erosion of coconut in Sri Lanka currently continues at a rate of 1% per annum (Peries

et al., 1992) as a result of fragmentation of land for industrial and/or urban development in the traditional coconut growing areas and natural disasters such as cyclones, droughts and diseases. In addition, the gradual replacement of the existing bio-diversity with a few improved varieties contribute to further narrowing of the genetic base. Furthermore, as a result of strict quarantine regulations banning importation of exotic coconut germplasm to Sri Lanka (due to the risk of introduction of lethal diseases such as Cadang-Cadang (Randles & Imperial, 1984) and Foliar decay (Randles et al., 1987)), a systematic programme for the genetic conservation of coconut within Sri Lanka is necessary to ensure continued access to new sources of genetic variation. As a result, collection and conservation of coconut bio-diversity in Sri Lanka was initiated in 1984 (Wickramarathne, 1984) with the objectives of preserving all actual or potentially valuable alleles including those which may be rare or geographically restricted. To date about 70 coconut populations of the variety *Typica*, form *typica*, have been collected by island-wide surveys based on passport data, morphological, physiological and quantitative data and representing various agro-ecological zones and soil types (Perera et al., 1996). Priority was also given for populations believed to have undergone preferential selection.

A minimum of 100 palms were randomly sampled from each site in the case of random sampling as 50–100 samples were considered more than adequate under most circumstances in capturing at least one copy of each allele occurs at frequency higher than 0.05 (Marshall & Brown, 1975). The number of palms sampled was restricted to either 100 or to the maximum number of palms identified in a site in the case of biased sampling strategy. However, the data used to prioritize the collections may exhibit considerable phenotypic plasticity. Despite these facts, however, similar coconut conservation strategies are being used in Indonesia, Philippines, India and Papua New Guinea (Medosa & Balingasa, 1978; Santos, 1983; Liyanage, 1977; Bhaskara Rao & Pilliai, 1982; T. Ovasuru, Personal communication).

As the characterization of this material is based on phenotypic data that may be influenced by environmental factors it is important to evaluate these materials at the DNA level to provide complementary information. A continued phenotypic and molecular evaluation of diversity will facilitate the formulation of conservation strategies to identify populations that represent core-germplasm collections for *ex situ* and *in situ* conservation. This is particularly important for tree crops like coconut because very large number of samples obtained by random sampling require large area for the conservation as living specimens.

Simple sequence repeats (SSRs) or microsatellites (Powell et al., 1996a) provide an ideal tool for such studies due to their high information content, ease of genotyping through PCR, co-dominant and multiallelic nature and high discriminating power (Morgante & Olivieri, 1993; Powell et al., 1996a, 1996b; Russell et al., 1997). In addition only small amounts of DNA are required and the quality of the DNA need not be as high as for most of the other DNA assay methods (Rafalski et al., 1996). Microsatellites have been used in both agricultural and breeding studies as well as in the analysis of natural plant populations (see Powell et al., 1996a and references therein) and have previously been shown to be appropriate for evaluating and characterizing coconut germplasm (Perera et al., 1999). The aim of the present investigation was to assess the levels and distribution of genetic diversity within and between populations of Sri Lanka tall coconut, variety *Typica,* form *typica.*

Methods and materials

Plant materials

Leaf materials were collected from 33 coconut populations from variety *Typica* form *typica* represented by 10 randomly selected palms (10 × 33 = 330 palms), planted in the *ex situ* coconut gene bank at the Coconut Research Institute of Sri Lanka for the SSR assay. The populations Clovis, Margeret, Dickwella, Mirishena and Akurassa were believed to be introductions.

DNA isolation and SSR analysis

DNA was extracted from fresh young coconut leaves using a modified CTAB method (Doyle & Doyle, 1987). SSR analysis was performed as described by Perera et al. (1999). The primer sequences and associated information are given in Table 1. Reaction products were separated on 6% polyacrylamide gels in 1x TBE buffer and visualized by autoradiography.

Data analysis

Diversity values based on allele frequencies were calculated for each nuclear SSR locus using Nei's unbiased statistic (1987).

Table 1. Primer information, gene diversity, number of alleles detected, and population differentiation statistics (F_{ST}) for eight coconut microsatellite primers

Locus	Repeat	Primer (5′–3′)	Size range (bp)	Gene diversity	Number of alleles	F_{ST}	*F_{ST}
CAC2	$(CA)_{12}(AG)_{14}$	AGCTTTTTCATTGCTGGAAT CCCCTCCAATACATTTTTCC	210–254	0.8461 ± 0.00053	9	0.051***	0.031***
CAC3	$(CA)_{13}$	GGCTCTCCAGCAGAGGCTTAC GGGACACCAGAAAAAGCC	187–203	0.6882 ± 0.0132	5	0.078***	0.013 ns
CAC4	$(CA)_{19}(AG)_{17}$	CCCCTATGCATCAAAACAAG CTCAGTGTCCGTCTTTGTCC	182–216	0.7486 ± 0.0105	9	0.049***	–0.005 ns
CAC6	$(AG)_{14}(CA)_9$	TGTACATGTTTTTTGCCCAA CGATGTAGCTACCTTCCCC	150–168	0.7315 ± 0.0095	7	0.061***	0.029***
CAC8	$(AG)_{10}(CA)_9$	ATCACCCCAATACAAGGACA AATTCTATGGTCCACCCACA	188–210	0.6768 ± 0.0129	9	0.024***	0.001 ns
CAC10	$(TA)_6CATA(CA)_{11}(TA)_8$	GGAACCTCTTTTGGGTCATT GATGGAAGGTGGTAATGCTG	195–205	0.4260 ± 0.0221	4	0.014***	0.014***
CAC13	$(CA)_9(TA)_5A(TA)_4(CA)_6$	GGGTTTTTTAGATCTTCGGC CTCAACAATCTGAAGCATCG	158–172	0.5167 ± 0.0082	3	0.056***	0.0176 ns
CAC56	$(CA)_{14}$	ATTCTTTTGGCTTAAAACATG TGATTTTACAGTTACAAGTTTGG	144–168	0.8221 ±0.0021	10	0.052***	0.028***
			Mean:	0.6820 ± 0.1446	7		
			Overall	0.9994 ± 0.0001	56	0.054***	0.015***

* F_{ST} – F_{ST} values calculated without introduced populations.

SSRs are assumed to follow a stepwise mutation model in comparison to the infinite allele mutation model (Valdes et al., 1993; Di Rienzo et al., 1994). The basic idea of the stepwise model is that mutations create new alleles that differ from their previous state by an increase or decrease of one step in the number of repeats. As empirical evidence suggests that mutational changes are often of one repeat unit (Weber & Wong, 1993), the stepwise mutation model has recently been 'revisited' (Shriver et al., 1993; Valdes et al., 1993; Di Rienzo et al., 1994). Since the SSR data did not always appear to conform to a stepwise mutation model (Rossetto et al., 1999) and coconut SSR data seemed to follow the same trend (i.e. certain loci did not exhibit the characteristic symmetrical, unimodal allele distribution), genetic distances, (D_{PS}), were calculated based on the proportion of shared alleles (P_S), where D_{PS} = (1- P_S) using the program MICROSAT (Version 1.5; Eric Minch, Stanford University, USA). P_S is the number of shared alleles summed over loci / (2x number of loci compared). In addition, it has previously been shown that this distance matrix is most suitable for assessing genetic relationships between recently diverged taxa below the species level (Bowcock et al., 1994; Provan et al., 1999). Dendrograms were constructed using the NEIGHBOR and DRAWGRAM options in the PHYLIP software package (V3.57c; Joe Felsenstein, University of Washington, USA) using the unweighted pair-group method using arithmetic averages (UPGMA).

The same distance matrices produced were used to perform a hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992), essentially as described by Huff et al. (1993) using the program ARLEQUIN (Version 1.0). The number of permutations for significance testing was set at 1000 for all analyses.

Results

Microsatellite polymorphism

The eight coconut-specific microsatellite primer pairs produced a total of 56 alleles ranging from 3 alleles for microsatellite primer CAC13 to 10 alleles for microsatellite primer CAC56 (Table 1). The average number of putative alleles per locus was 7. Only

Table 2. Allele frequency data for 8 microsatellite loci studied

Locus	Allele	Allele frequency	No. of populations that the allele was identified	Allele type (considering only local populations)
CAC2	254 bp	0.086	19	Common, widespread
	252 bp	0.0391	15	Common, widespread
	250 bp	0.0485	14	Common, sporadic
	248 bp	0.1064	27	Common, widespread
	246 bp	0.0282	4 (3 in introductions)	Rare, localised
	240 bp	0.2254	32	Common, widespread
	234 bp	0.2003	32	Common, widespread
	232 bp	0.1878	32	Common, widespread
	220 bp	0.0782	22	Common, widespread
CAC3	203 bp	0.0413	16	Common, widespread
	201 bp	0.1774	31	Common, widespread
	199 bp	0.4847	32	Common, widespread
	197 bp	0.1682	30	Common, widespread
	187 bp	0.1284	28	Common, widespread
CAC4	216 bp	0.0125	8	Rare, sporadic
	212 bp	0.2665	32	Common, widespread
	208 bp	0.3934	32	Common, widespread
	204 bp	0.1332	25	Common, widespread
	200 bp	0.0752	25	Common, widespread
	192 bp	0.0047	3	Rare, sporadic
	190 bp	0.0016	1 (introduction)	–
	188 bp	0.0345	3 (all in introductions)	–
	186 bp	0.0705	16	Common, sporadic
CAC6	164 bp	0.0370	5 (all in introductions + EC)	–
	160 bp	0.0478	17	Common, widespread
	158 bp	0.0478	14	Common, sporadic
	156 bp	0.0247	10	Common, sporadic
	154 bp	0.3488	33	Common, widespread
	152 bp	0.3457	33	Common, widespread
	150 bp	0.1418	29	Common, widespread
CAC8	214 bp	0.0016	1	Rare, localised
	212 bp	0.0207	9	Common, sporadic
	210 bp	0.4815	33	Common, widespread
	208 bp	0.2600	31	Common, widespread
	204 bp	0.1541	29	Common, widespread
	202 bp	0.0048	1 (introduction)	–
	200 bp	0.0128	5	Common, sporadic
	198 bp	0.0594	20	Common, widespread
	188 bp	0.0048	2 (both in introductions)	–
CAC10	203 bp	0.0242	7 (two introductions)	Common, sporadic
	201 bp	0.1519	29	Common, widespread
	197 bp	0.7415	33	Common, widespread
	195 bp	0.0824	21	Common, widespread
CAC13	172 bp	0.0267	14	Common, widespred
	162 bp	0.4167	33	Common, widespread
	158 bp	0.5566	33	Common, widespread
CAC56	168 bp	0.0449	5 (all in introductions)	–
	166 bp	0.0561	5 (all in introductions)	–
	164 bp	0.0112	5 (all in introductions)	–
	160 bp	0.0898	21	Common, widespread
	158 bp	0.1573	30	Common, widespread
	156 bp	0.2584	28	Common, widespread
	154 bp	0.0112	5 (all in introductions)	–
	152 bp	0.0112	5 (all in introductions)	–
	146 bp	0.0112	5 (all in introductions)	–
	144 bp	0.3595	33	Common, widespread

Table 3. Classification of number of alleles according to their frequencies and distribution between populations considering only local populations

	Common alleles	Rare alleles	Total
Localised	0	2	2
Sporadic	8	2	10
Widespread	33	0	33
Total	41	4	45

three unique alleles (i.e. those found in a single population) were present, two in population Clovis (190 bp, CAC4 and 202 bp, CAC8) and one in population Moorock (214bp, CAC8). However their frequency was less than 5%. Out of 56 alleles detected, 11 alleles were restricted to the introduced populations (Clovis, Margeret, Dickwella and Mirishena). Allele frequencies across populations and number of populations in which a particular allele is present are given in Table 2. Alleles were classified according to frequency and distribution as outlined by Marshall & Brown (1975) Brown (1978). Frequency and distribution of the locally present 45 alleles identified in 28 populations are shown in Table 3. Alleles were categorised firstly as common and rare alleles; a common allele in this study was defined as an allele that was present in at least one population at a frequency greater than 5% and a rare allele as an allele that never occurs at frequency higher than 5%. Secondly alleles were categorised as either widespread, sporadic or localised, defined as an allele which was present in more than 12 populations, present in between 2 and 12 populations or present in only one population respectively. A total of 4 alleles were classified as rare alleles. Two of the rare alleles were sporadic and two were localised being present in populations Moorock and St. Annes. A total of 41 alleles were classified as common alleles out of which 33 were widespread and 8 were sporadic. No common localised alleles were found. Accordingly common widespread alleles account for 73.5% of the total alleles detected in the 28 local populations.

Gene diversity detected for each population across loci appeared to be high, mean gene diversity ranging from 0.526 for Debarayaya to 0.683 for Dickwella. The total gene diversity index of 0.999 indicates that most of the coconut palms are unique genotypes. The gene diversity for each locus across populations is given in the Table 1 and gene diversity was observed to range from 0.846 for locus CAC-2 to 0.426 for locus CAC10 with mean gene diversity of 0.682.

A dendrogram based on proportion of shared alleles (D_{PS}) is shown in Figure 1. Only five populations namely, Clovis, Margeret, Dickwella, Mirishena, and the *in vitro* constructed embryo culture population (*in vitro* drought screened) were clearly separated from the main group of populations.

The partitioning of variation within and between populations reflected as F_{ST} statistics is shown in Table 1. The F_{ST} statistic varies among loci from 0.014 to 0.078 with a mean of 0.054 (5.4%) which is highly significant reflecting a moderate level of population differentiation. The analysis was repeated excluding the 4 exotic populations and the embryo cultured population and average F_{ST} statistic was then 0.015 (1.5%) which is very much lower. However the value was statistically significant for population differentiation. The data were also analysed according to the SSR Stepwise Mutation Model (SMM) and the average R_{ST} value was observed as 0.138, which is statistically highly significant. The analysis was once again repeated excluding the 4 exotic populations and the embryo cultured population and average R_{ST} statistic was then observed as 0.01 (1%), which is again very much lower and statistically not significant. Accordingly the within population variation of local coconut populations accounted for 99% of the observed variation.

Discussion and conclusions

Although large populations of cross-pollinating species are likely to contain a high proportion of the total genetic variation within populations, it is unlikely that any single population will contain all of the genetic variation. The total genetic variation of a species is therefore likely to be distributed among populations as the impact and direction of natural selection varies from one to another, due to environmental variation and genetic drift (Lawrence & Rajanaidu, 1985). Therefore with germplasm conservation programmes, it is imperative to accurately measure the amount of genetic diversity and its distribution within and between populations. To these ends, molecular markers provide an efficient and unbiased estimate of these statistics, free of environment effects. The microsatellites used in this study appeared to possess a significant potential in this respect.

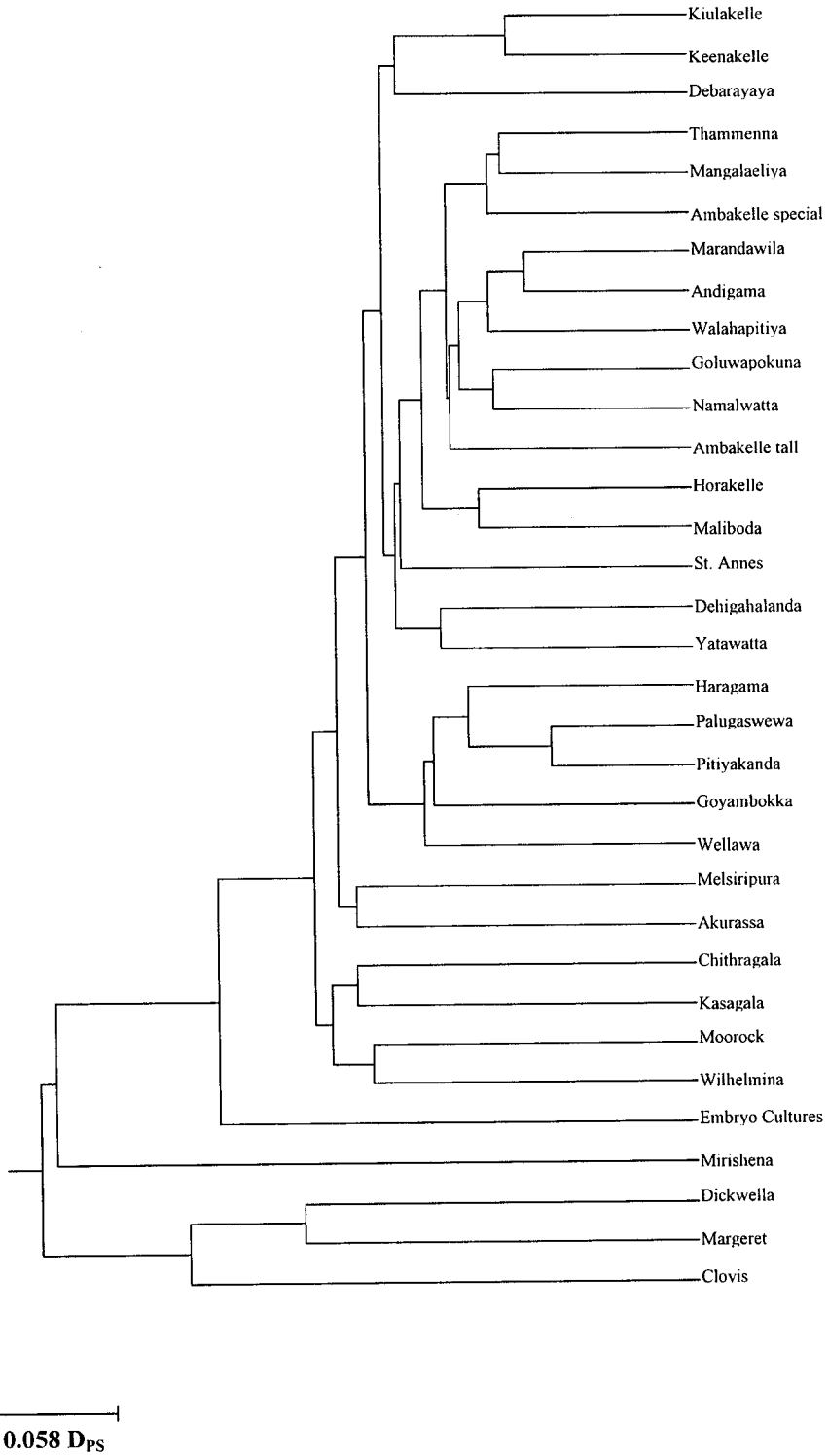

Figure 1. The dendrogram based on D_{PS} genetic distances showing the genetic relationships between accessions.

The graphical representation of the genetic distances between populations presented as a phenetic tree clearly separated the five populations, Clovis, Margeret, Dickwella, Mirishena and the embryo-cultured population from the rest of the populations. This was further evident by the allelic distribution with alleles being shared in these populations which are absent from the rest of the populations. The passport data for the original populations of Clovis, Margeret, Dickwella revealed that these populations are exotic and are early introductions. They are also morphologically different from the rest of the populations and resemble the variety San Ramon from the Philippines in their nut shape and trunk characteristics (unpublished field observation). The passport data revealed that the population Mirishena was also identified by the local people in the area as an exotic type. They are locally named as 'naw-pol' meaning brought by ships.

Total genetic diversity was partitioned between populations using an analysis of molecular variance (AMOVA) procedure. The same methodology has also been used in partitioning the genetic diversity of coconut in the previous study with AFLPs (Perera et al., 1998) where there is an approximately equally distributed variation between and within forms of tall coconuts. The results of the AMOVA (Table 1) in this study show a very high percentage of within population variation (98.5%) for the tall coconut form *typica*. A similar observation has been made in various other out-crossing tree species (Hamrick et al., 1992; Chung & Kang, 1994; Leonardi & Menozzi, 1995; Nesbitt et al., 1995; Yeh et al., 1995; Maguire & Sedgley, 1997; White & Powell, 1997; Soranzo, 1999). However this is a general observation in long-lived wind pollinated tree species (Hamrick & Godt, 1990).

Coconut being a predominantly insect pollinated out-crossing perennial species, it is unlikely that gene flow via pollen would account for the high level of within population variation because species that rely on insects or animals for pollination and dispersal of genes generally display stronger genetic structure (Hamrick et al., 1992). Therefore the low level of differentiation may be because of the common history of native Sri Lankan tall coconuts and the long generation time. The preferential selections that had been in operation in some of these populations may not have been effective, as the selection procedure involved selection of superior mother palms followed by use of open pollinated nuts from those mother palms for the subsequent planting without eliminating the unselected palms. The contribution of alleles from the unselected palms as pollen donors to the second generation probably diminishes the effect of selection. It has been shown that moderate levels of gene flow are able to balance the effects of factors responsible for population differentiation such as genetic drift and directional selection (Slatkin, 1987). In contrast, Ashburner et al. (1997) who reported the use of RAPDs to study the genetic diversity of 17 distinct South-Pacific coconut populations observed approximately 40% between population variation. This observation is contrary to the observation made in Sri Lanka where populations within a single isolated island are involved. The 60% of the within population variation observed by Ashburner et al. (1997) indicates that there is a considerable level of gene flow between the populations while other genetic phenomena are responsible for the 40% of the population differentiation. This can be explained as possible common history of coconuts between isolated islands and possible strong founder effects with subsequent selections by local human inhabitants. Alternatively higher levels of within population variation (60%) may also be explained as result of the variable influence of gene migration between South Pacific populations in isolated small islands via both pollen and seed flow, but mainly via seeds floating between islands.

The classification of alleles according to their frequencies and distribution shows that out of a total of 45 alleles, only 2 alleles appeared to be rare and localised, which can be considered as the most difficult type of alleles to capture in any kind of sampling strategy. In addition 2 and 8 alleles were grouped as rare sporadic and common sporadic, hence capturing of these alleles depends on the number of sites sampled. The majority of alleles (33) were common and widespread indicating that various sampling strategies; more sites and fewer trees per site or fewer sites and more trees per site would easily sample these alleles (Marshall & Brown, 1975). There were not any alleles falling into the category common localised. Marshall & Brown (1975) argue that rare alleles are probably low in adaptive value and are of less interest to breeders. Therefore the aim of collection strategies should be to collect at least one copy of each allele occurring with a frequency of at least 0.05. The observations that a high percentage of common widespread alleles ($>$70%) and the very high level of within population variation (98.5%), suggests that a single large random collection from the whole target area would capture most of the desirable genetic variation present within Sri Lanka tall coconut variety *Typica* form *typica*.

The populations Moorock, that contains 33 alleles and populations Melsiripura, Dehigahalanda, Debarayaya and Keenakelle all together seem to have captured all allelic variability detected and hence appear theoretically as a core-collection for the *ex situ* genebank. Further it can be concluded that the existing *ex situ* genebank adequately represents the target collection area and hence further random collections would not be necessary. However it would be worthwhile conducting targeted collections for traits such as drought and disease resistance and from any special ecological niche. These results also suggest that consideration be given to passport data since this would clearly help in identifying exotic germplasm which had been already introduced, and which could be introduced into the breeding programme to maximise potential heterosis without the risk of the introduction of lethal disease. The populations Clovis, Margeret, Dickwella and Mirishena were shown to be the populations with the highest average genetic distance. These findings suggests that the genetic base of coconuts in Sri Lanka is restricted and highlights the need to utilise exotic germplasm in the breeding programme for increased hybrid vigor.

All the populations studied here exhibited a high level of genetic diversity indicating a highly heterogeneous nature of coconut. The eight SSR primers uniquely discriminated all the 330 genotype studied and this indicates that each individual palm of coconut is a unique genotype. In general the high level of diversity detected together with the high level of morphological and quantitative variation between palms suggests further scope for improvement of coconut population in Sri Lanka through mass selection, preferably followed by controlled pollination to ensure high selection differential.

In summary the study based on SSRs revealed a very low level of population differentiation. Therefore, a single large random collection from the whole target area would capture most of the variation found in the Sri Lankan tall coconut variety *Typica* form *typica*. Further the existing *ex situ* genebank adequately represents the target collection area and hence further random collections would not be necessary. This study highlights the importance of utilising exotic germplasm in the breeding programme.

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