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Genetic Relationships of Indigenous King Coconut (*Cocos nucifera* L.) Populations as Determined by SSR Markers

M. K. Meegahakumbura[1,2*], M. T. N. Indrachapa[3], T. S. Baddegama[2], M. M. Hettiarachchi[2], H. D. M. A. C. Dissanayaka[2], C. R. K. Samarasinghe[2], P. R. Weerasinghe[2] and P. N. Dasanayake[4]

[1] Department of Export Agriculture, Uva Wellassa University, Badulla, Sri Lanka
[2] Genetics and Plant Breeding Division, Coconut Research Institute, Lunuwila, Sri Lanka
[3] Tissue Culture Division, Coconut Research Institute, Lunuwila, Sri Lanka
[4] Department of Botany, University of Sri Jayawardenepura, Colombo, Sri Lanka

* Email: muditha@uwu.ac.lk

Abstract

King Coconut (KC) is an indigenous and highly valuable germplasm resource in Sri Lanka. Yet, KC populations' genetic diversity, relationships, and conservation strategies are not fully understood. Indigenous old KC populations have been dispersed across a few ancient villages in Sri Lanka. Five geographically dispersed locations were selected in Sri Lanka, and 20 KC individuals from each location were collected for the current study. Six randomly selected samples from every geographical location, together with the standard two Sri Lanka Red Dwarf and two Sri Lanka Tall varieties were initially genotyped using 21 SSR markers for polymorphism. Based on the results, ten polymorphic SSR markers were selected and used for genotyping. Power Marker, STRUCTURE, and GenAlex software were used for the SSR genetic analysis. Results revealed 0.62 mean Gene Diversity (Hs), 4.2 mean allele number, and 0.55 polymorphic information content (PIC). Clear differentiation of populations was observed with the STRUCTURE and UPGMA dendrogram. Single branching in the UPGMA dendrogram for Anuradhapura and Marandawila KC populations revealed high genetic uniformity over multi-branched Kadugannawa and Colambageara populations. According to AMOVA, 64% of the genetic variation has been partitioned among populations, indicating moderate population differentiation. Detail analysis, including a higher number of KC populations and systematic molecular analysis using more SSRs/SNPs needed in the future before implementing conservation and utilization strategies.

Keywords: *Cocos nucifera*, King Coconut, SSRs

Introduction

King Coconut is regarded as an indigenous coconut variety in Sri Lanka (Attanayake and Fernando, 1987). Due to its bright-orange-colour epicarp and delicious nut water taste, 6.4 million King Coconuts were exported in 2019 generating US$3.9 million in income for the Sri Lankan economy (Nainanayake, 2019). Despite the decreasing number of King Coconuts exports due to the spread of COVID-19, income from its exports increased to US$4.5 million in 2021.

King Coconut (KC) is a semi-tall variety and it is categorized under the variety Aurantiaca that exhibits intermediate morphological characteristics between the tall (Typica) and Dwarf (Nana) categories of coconut (Liyanage, 1958). The tall coconuts are highly allogamous while the Dwarfs and KC are predominantly autogamous (Liyanage,1958). Therefore, low genetic diversity and poor population differentiation are hypothesized within KC although a considerable diversity among qualitative characteristics can be seen within the KC population. Nevertheless, a considerable amount of genetic diversity can be expected through accidental cross-pollination since the chance for self-pollination depends on the length of the male and female phases for their overlapping.

During the mid-1980s, the Coconut Research Institute of Sri Lanka (CRISL) distributed KC seedlings among growers to popularize the crop. Seedlings used in this programme would be coming from a few populations and hence, there is a possibility to record a narrow genetic diversity of the KC germplasm in the regions where these coconut seedlings were distributed. KC plants maintained for longer than 40-50

Table 1. Geographical locations of KC populations selected for the study

No	Population	Population Code	District	Agroecological zone	Location	Remarks
1	Pannala	p	Kurunegala	Intermediate	7`18``N, 80`85``E	Population was collected from a home garden
2	Colambageara	C	Ratnapura	Intermediate	6`43``N, 80`76``E	Population was collected from multiple home gardens surrounding the Sankapala ancient Buddhist temple
3	Maradhawila	M	Kurunegala	Intermediate	7`53``N, 79`87``E	Population was collected from Marandawila NLDB farm
4	Anuradhapura	A	Anuradhapura	Dry	8`33N 80`50``E	Total population was collected from a private estate
5	Kadugannawa	K	Kandy	Wet	7`25``N 80`52``E	Population was collected from multiple home gardens surrounding the Walagamba RajamahaViharaya

years in remote villages and temples may carry a considerable genetic diversity. Due to old age, urbanization, land fragmentation and replanting improved commercial coconut cultivars, these valuable KC populations are under the threat of genetic erosion. In addition, KC is highly susceptible to white fly infestations. With the serious white fly outbreaks spreading throughout Sri Lanka, KC germplasm is further challenged to reduce its vigour and weaken the palms (Karunarathne et al., 2018). Therefore, systematic studies on genetic diversity, genetic relatedness, nut-water quality and conservation efforts are the needs of the hour.

Molecular markers such as Simple Sequence Repeats (SSRs) are ideal tools for studying crop genetic diversity and relatedness (Powell et al., 1996). The genetic diversity of coconut germplasm in Sri Lanka and across the globe has extensively been studied using SSRs (Perera et al., 1999; Perera et al., 2003; Rivera et al., 1999; Gunn et al., 2011; Perera et al., 2016; Riangwong et al., 2020). Although a few KC samples have been included in some of these previous studies (Perera et al., 2001; Dassanayaka et al., 2005; Dassanayaka et al., 2009), the genetic diversity and population structure of KC have not been fully investigated.

In the current study, five in situ KC populations were selected, to investigate the genetic diversity and genetic relationships of KC germplasm.

Materials and Methods

Five old King Coconut (*Cocos nucifera* L.) populations (>40 years) were selected from Pannala, Anuradhapura, Marandawila, Kadugannawa and Colambageara areas in Sri Lanka and they represent distinct geographical locations and agroecological zones (Wet zone, intermediate zone, and dry zone) of Sri Lanka (Punyawardena, 2008). Individual KC palms collected from a single geographical location are referred to as a population. In the present study, geographically dispersed KC populations were selected while recording the GPS locations and the description of sample locations is given in Table 1. Although KC germplasm is predominantly self-pollinated, accidental out-crossing of KC with Sri Lanka Tall has been reported and this natural hybrid is referred to as "Nipol" in the local language (Perera et al., 2015). "Nipol" could be clearly identified morphologically as the nuts colour of these hybrid palms changes from orange to brown/green colour. In the current study, such off-type palms were eliminated and distinct morphological features of nuts (colour, shape), fronds and trunk (Liyanage, 1958) were used to identify KC palms and each population was represented by 20 individuals.

Genotyping was carried out at the molecular biology laboratory of the Genetics and Plant Breeding Division, Coconut Research Institute, Sri Lanka. Two Sri Lanka Tall palms and two Sri Lanka Red Dwarf palms were used as standards (Indrachapa et al., 2018).

DNA Extraction and SSR analysis

DNA was extracted from the fresh bud leaves of collected KC palms and standard palms using the modified CTAB method (Perera et al., 1999). DNA was quantified using the Nanodrop 1000 (Thermo Fisher).

A total of 21 primer pairs were used for polymorphism screening. These primers were; 13 Cncir (Baudouin and Lebrun, 2002), 4 CAC (Perera et al., 1999), and 4 CNZ (Rivera et al., 1999). The annealing temperatures of the primers were slightly modified during the PCR optimization process.

PCR amplification was carried out initially with the selected six samples from all five populations together with four standard samples for all 21 primers to select polymorphic SSR markers (Table 2). The PCR reaction was performed in a 10µL reaction mixture containing 1 × PCR buffer containing 2 mM $MgCl_2$, deoxynucleoside triphosphates (0.35 mM each; Qiagen), 0.6 µM of each primer (Integrated DNA technology-IDT), 0.8U

Table 2. SSR primers used for genotyping of KC populations

Serial No.	SSR Primers	Forward sequence	Reverse sequence	Annealing temperature
1	Cncir A3	AATCTAAATCTACGAAAGCA	AATAATGTGAAAAAGCAAAG	49°C
2	Cncir B6	GAGTGTGTGAGCCAGCAT	ATTGTTCACAGTCCTTCCA	53°C
3	Cncir C7	ATAGCATATGGTTTTCCT	TGCTCCAGCGTTCATCTA	47°C
4	Cncir C12	ATACCACAGGCTAACAT	AACCAGAGACATTTGAA	45°C
5	Cncir E12	TCACGCAAAAGATAAAACC	ATGGAGATGGAAAGAAAGG	59°C
6	Cncir F2	GGTCTCCTCTCCCTCCTTCTCTA	CGACGACCCAAAACTGAACAC	63°C
7	Cncir H4	TTAGATCTCCTCCCAAAG	ATCGAAAGAACAGTCACG	51°C
8	CAC 65	GAAAAGGATGTAATAAGCTGG	TTGTCCCCAAATATAGGTAG	53.5°C
9	CAC 68	AATTATTTTGGCGTTACATGCATC	AACAGCCTCTAGCAATCATAG	54°C
10	CNZ 29	TAAATGGGTAAGTGTTTGTGC	CTGTCCTATTTCCCTTTCATT	53°C

Taq polymerase (GoTaq- Promega) and 3µL (25ng/µL) of DNA from each selected sample. Amplification was performed in an Applied Bio-systems thermal cycler. The temperature profile varies with an initial denaturation at 94°C for 5 minutes followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature of each primer, 1 minute at 72°C and a final cycle of 5 minutes at 72°C. Out of 21 primers, 10 polymorphic primers were selected for the detailed study (Table 2). PCR amplifications were carried out with 100 DNA samples and 4 standards.

PCR products were subjected to 6% denaturing polyacrylamide gel electrophoresis and visualized by silver staining (Anolles and Petter, 1994). The bands were compared and scored visually with the 100bp DNA ladder (Qiagen; Gelpilot).

Data analysis

Alleles amplified by SSR markers were scored under a lightbox and the scored data were analyzed using PowerMarker version 3.25 (http://www.powermarker.net). The total number of alleles, the number of alleles per locus, gene diversity and the polymorphism information content (PIC) were tabulated. The genetic distance was estimated by cluster analysis. The dendrogram was constructed using genetic distances among individuals derived by Nei's method (Nei et al., 1983) in PowerMarker software (Liu and Muse, 2005). Tree View (version 1.6) was used to visualize the dendrogram (Page, 2002).

Population structure was analyzed using multi-locus genotypic data in the software program STRUCTURE version 2.3.4 (Pritchard et al., 2000). This software uses an interactive algorithm to allocate individuals (probabilistically) into K clusters, based on the clustering method and assumes a model in which there are K populations. The data were analyzed using the admixture model and each run was done for K = 1 to K = 10, 100,000 burn period and 100,000 MCMC repeats. Graphical representations of STRUCTURE results were produced using the DISTRUCT (Rosenberg, 2004).

The analysis of molecular variance (AMOVA) was performed to dissect the genetic variation among individuals by the GenAlex (version 6.5), testing Fst by 999 random permutations (Peakall and Smouse, 2006).

Results and Discussion

Genetic Diversity

All the 21 primers used in the primary screening generated PCR products. However, 11 primers generated monomorphic banding patterns and were hence excluded from the study. The remaining ten polymorphic primers were used to genotype 100 KC with the standard varieties. Selected primer pairs generated 42 alleles, averaging 4.2 alleles per locus ranging from three alleles for Cncir E12, while six alleles for Cncir B6. All 104 samples showed a mean Gene diversity of 0.6241. Higher Gene diversities were predominant among CNZ 29 and Cncir C7 loci. The polymorphism information content (PIC) is a closely related diversity measure. The mean PIC value of all ten SSR was 0.5536. PIC values were high for Cncir C7, Cncir F2 and CAC 65 loci (Table 3).

Despite its importance, KC germplasm has not been studied in detail in the past owing to its self-pollination nature and it was hypothesized that the KC germplasm exhibits low genetic diversity. As a result, only a few KC samples (less than five) have been incorporated into previous studies to understand their systematic position (Perera et al., 2001; Dassanayaka et al., 2005; Dassanayaka et al., 2009). Therefore, geographically dispersed KC populations were selected for the current study to investigate the existing genetic diversity among KC populations in Sri Lanka. Even in the current study, eleven out of 21 SSR primers (52%) were found to be monomorphic and hence omitted from the detailed analysis. Ten polymorphic primers used recorded the existence of 3-6 different alleles in KC germplasm, indicating a moderate level of genetic variation (Table 3). The mean number of alleles found in cross-pollinating tall coconut germplasm in Sri Lanka has been reported as seven alleles (Perera et al., 2001)

Table 3. Number of Alleles, Major allele frequency, Gene Diversity and Polymorphism Information Content (PIC) for ten SSR loci

No.	SSR locus	No. of alleles	Major allele frequency	Gene diversity (H_s)	(PIC)
1	Cncir E12	3	0.5269	0.5513	0.4549
2	Cncir B6	6	0.5281	0.5880	0.5127
3	Cncir C7	4	0.3750	0.6913	0.6323
4	Cncir H4	4	0.5745	0.5185	0.4183
5	CAC 68	4	0.4082	0.6520	0.5822
6	Cncir C12	4	0.5824	0.5775	0.5175
7	Cncir F2	4	0.3776	0.6903	0.6310
8	CAC 65	5	0.3723	0.6766	0.6124
9	Cncir A3	4	0.5435	0.6032	0.5389
10	CNZ 29	4	0.4074	0.6923	0.6358
	Mean	4.2	0.4696	0.6241	0.5536

Table 4. Analysis of the molecular variance (AMOVA) of the KC populations

Source	df	SS	MS	EV	%	F statistis		Probability
Among populations	4	410.005	102.501	2.497	64%	**Fst**	0.636	0.001
Among Indiv	95	247.450	2.605	1.177	30%	**Fis**	0.825	0.001
Within Indiv	100	25.000	0.250	0.250	6%	**Fit**	0.936	0.001
Total	199	682.455		3.925	100%			

df, degree of freedom; SS, sum of squared observations; MS, mean of squared observations; EV, estimated variance.

while, it was 3.3 alleles in self-pollinating Yellow Dwarf (Kamaral et al., 2016). In the present study, the KC germplasm, which is morphologically intermediate between talls and Dwarfs, reported 4.2 mean alleles. The current study reported 0.62 mean gene diversity (H_s) and 0.5536 Polymorphic information content (PIC) which is higher than the respective values of 0.37 and 0.3236 reported for Sri Lanka Yellow Dwarf germplasm (Kamaral et al., 2016).

Genetic Relationships

The graphical representation of the genetic relationship between five KC populations and four standard samples is presented in the UPGMA phylogenetic tree (Figure 1). UPGMA tree exhibits a major group containing 98 KC, two Red Dwarf samples, an out-group comprising two Sri Lanka Tall samples (TT1, TT2), and two KC samples c1, c2 collected from Colambageara. The main cluster was comprised of two sub-clusters. One sub-cluster consists of Anuradhapura and Marandawila population clades and two Red Dwarf samples formed an out-group. Other sub-cluster consists of Pannala, Colambageara, and Kadugannawa population clades. Anuradhapura and Marandawila populations exhibited higher uniformity whereas Colambageara, and Kadugannawa populations exhibited comparatively a higher variation with multiple branching (Figure 1).

Genetic relationship studies of the Sri Lankan coconut germplasm indicated that KC was closely related to Sri Lankan dwarfs (Perera et al., 2001; Dassanayaka et al., 2005; Dassanayaka et al., 2009). The clustering of two Red Dwarf samples with KC germplasm and out-grouping of Sri Lankan tall from the major clade further confirms previously reported results. However, 2 samples collected from the Colambageara KC population (c1 and c2) have been grouped with the tall samples. These two samples might have been misidentified or possibly naturally hybridized with tall and later backcrossed with KC, thereby showing morphological resemblance. Five KC populations were initially divided into two sub-clusters and further divided into five population clades indicating a differentiation among populations. Three populations (Pannala, Anuradhapura and Marandawila) were collected from a single home garden/estate showing a close genetic similarity within the population. Therefore, all the samples might have originated from a single germplasm source. However, the other two populations (Colambageara and Kadugannawa) collected from multiple home gardens surrounding ancient Buddhist temples showed higher diversity (Figure 1). This indicates multiple introductions of KC samples possibly from different germplasm sources. Offering Coconut/King Coconut seedlings for Buddhist temples was an ancient tradition in Sri Lanka and this might have led to the higher differences exhibited in Colambageara and Kadugannawa populations.

Population structure analysis

Results of the STRUCTURE analysis with the "admixture model" are given below in Figure 2 showing K as 2, 3, 4 and 5

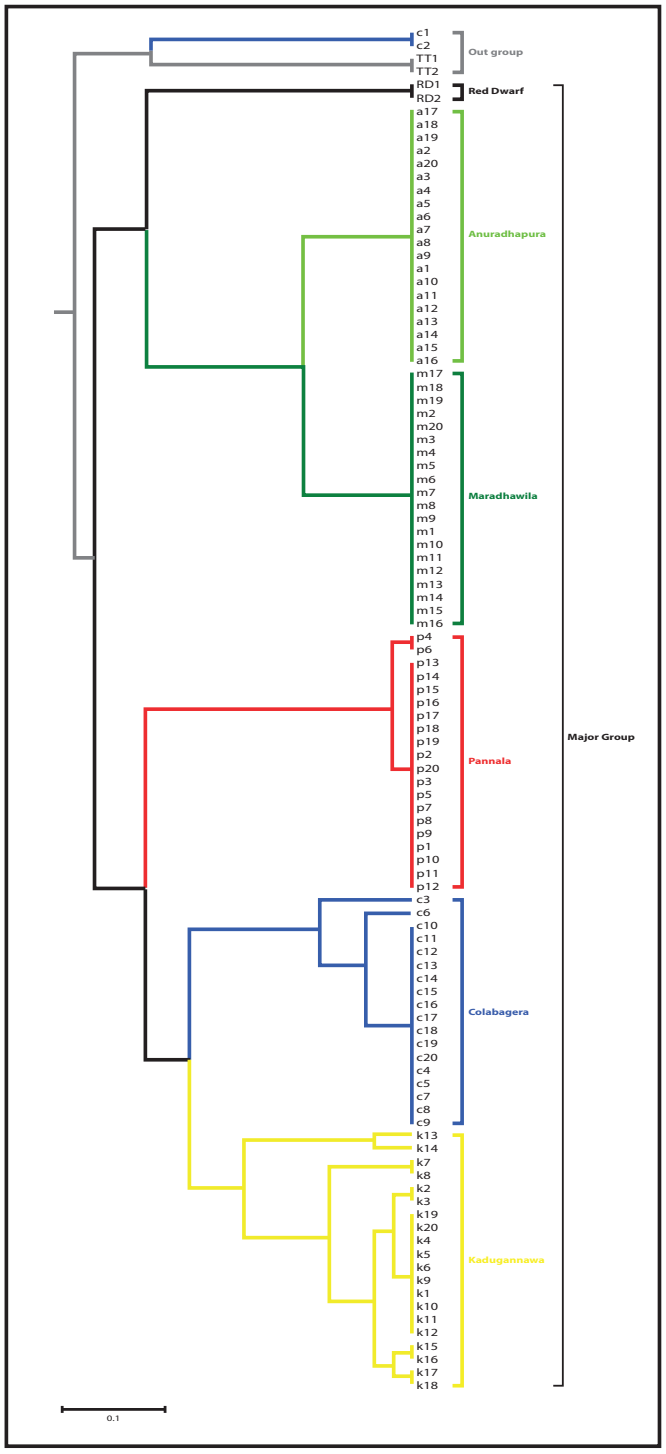

Figure 1. UPGMA phylogenetic tree of five King Coconut populations and standard coconut varieties. Light green represents Anuradhapura KC population; Dark green represents Marandawila KC population; Red represents Pannala KC population; Blue represents Colambageara KC population and Yellow represents Kadugannawa KC population respectively. Ash colour denotes two standard tall coconut varieties and Black for two Red Dwarf coconuts. The coloring in the legend represents the KC population and standard coconut samples.

respectively. When K=2, all KC samples from five populations were clustered into two groups. One represented KC samples collected from Pannala, Colambageara and Kadugannawa and the other represented KC samples from Marandawila and Anuradhapura. When K=3, Pannala population formed a separate cluster while Colambageara and Kadugannawa populations remained similar to K=2. Marandawila and Anuradhapura populations also formed a single group. At K=4, Colambageara and Kadugannawa populations were further separated into two clusters. The Pannala population too formed a separate cluster. Marandawila and Anuradhapura populations remained similar to K=2 and K=3. With K= 5, no additional distinct genetic groups were formed, although the group heterogeneity increased (Figure 2). These results together indicate that K=4 captures most of the structure in the data and seems biologically sensible.

Cluster analysis with the STRUCTURE is a powerful tool to define genetically distinct groups. Kamaral et al. (2016) used the STRUCTURE analysis to reveal the population structure of the Sri Lankan Yellow Dwarf coconut germplasm. In the current study, 100 KC samples from five distinct populations were divided into four groups at K=4 (Figure 2). At K=5 no new groups were formed hence, K=4 was recognized as the best clustering solution for the current KC study.

At K=4, Pannala, Colambageara and Kadugannawa populations were clearly separated into three groups similar to the UPGMA tree. However, Marandawila and Anuradhapura populations remained intact. Interestingly, Pannala and Marandawila populations were both located in Kurunegala District and in comparatively close proximity to one another (Figure 1). Yet, multiple analyses with STRUCTURE and UPGMA trees have differentiated these two populations. On the other hand, Marandawila and Anuradhapura populations were located around 140 km apart in the Kurunegala and Anuradhapura Districts respectively. The clustering of these two populations together in the STRUCTURE and UPGMA tree resembles close genetic affinity (Figure 1; Figure 2). Marandawila is a well-known National Livestock Development Board (NLDB) owned estate belonging to the government of Sri Lanka where coconut has been planted over a very long period starting from the British era. Anuradhapura KC population was comparatively younger than the Marandawila. Therefore, there are possibilities that KC germplasm might have been directly or indirectly exchanged between these two locations. However, fingerprinting with ten SSR primers is barely sufficient to come up with strong conclusions. This study could be repeated with a higher number of polymorphic SSRs (Xia et al., 2014; Riangwonget al., 2020).

Although, KC populations revealed a clear structure similar to the Sri Lankan Yellow Dwarf (Perera et al., 2016), cross-pollinating Sri Lankan tall types are considered as a single large population as clear genetic grouping could not be seen among tall populations (Perera et al., 2001). Predominantly self-pollination with rare intra-varietal and inter-varietal hybridization in Dwarf and KC might have resulted in the clear structure compared to out-crossing talls. Selecting only the polymorphic SSR primers for the detailed KC genetic analysis

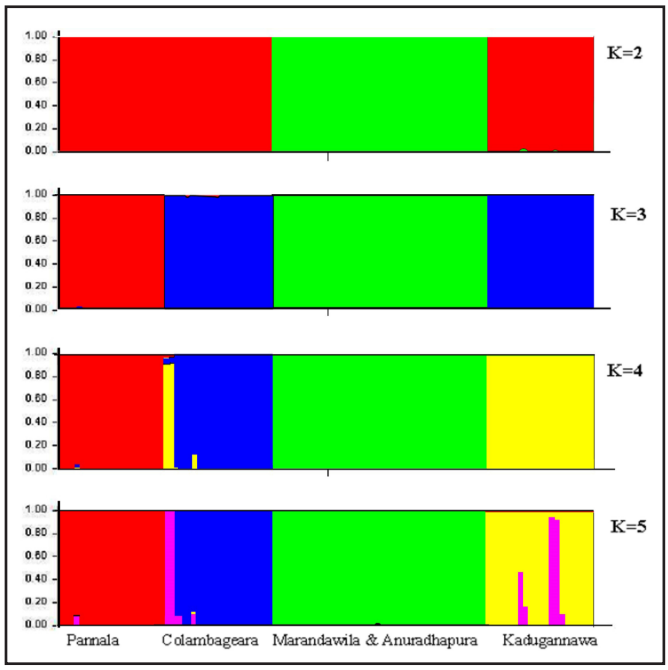

Figure 2. Results of the STRUCTURE analysis at K= 2 to 5 for a total of 100 King Coconut samples collected from Pannala, Colambageara, Marandawila, Anuradhapura and Kadugannawa, Sri Lanka

is possibly responsible for the clear clustering pattern shown in the UPGMA dendrogram.

Analysis of Molecular Variance (AMOVA)

Analysis of Molecular Variance (AMOVA) was presented here to illustrate the genetic differentiation among the KC populations in Sri Lanka and the results are given in Table 4. According to the results, 64% of the total variability was partitioned among KC populations ($P<0.001$), while 30% of the total variability was partitioned among individuals and 6% within individuals ($P<0.001$). Huang et al. (2019) have shown that 91.7% of genetic variation was partitioned among populations in self-pollinating ginger (*Zingiber corallinum*) indicating a high population differentiation. In contrast, in extensive cross-pollinating crops like tea, 95% of the genetic variation was partitioned within populations (Zhao et al., 2021) indicating a poor population differentiation. In the current study, a moderate level of genetic differentiation was observed among KC populations.

Future Directions

As we have reported the existence of genetic diversity, population structure and moderate genetic differentiation for geographically dispersed KC populations, comprehensive studies on closely distributed populations are recommended to carry out in future. These studies possibly offer vital inferences on highly diverse populations and diversity hotspots for KC germplasm conservation efforts. Detail marker systems such as Single Nucleotide Polymorphisms (SNPs) could also be used to reveal a comprehensive understanding of genetic diversity and relationships of indigenous KC germplasm (Nimmakayala et al., 2014; Meegahakumbura et al., 2018). Apart from genetic diversity, nut water quality parameters and other qualitative traits also needed to be considered before planning the KC germplasm conservation strategies for Sri Lanka.

In addition, King Coconut germplasm is also available in Oman, however, there are clear records that it was imported from Sri Lanka in 1983 (Perera et al., 2012). If a few leaf/DNA samples could be included from Oman in the genetic relationship study with KC populations from Sri Lanka, we could identify from which populations the germplasm was introduced to Oman. Although KC is regarded as an indigenous germplasm in Sri Lanka, similar morphological forms have also been observed in Tanzania and Papua New Guinea. Genetic relationships between these similar morphological forms and king coconut have never been attempted. Therefore leaf/DNA samples also needed to be collected from Tanzania and Papua New Guinea to compare their genetic relationship with the KC germplasm in Sri Lanka. This would be an interesting biogeographic or human-mediated coconut germplasm exchange study. Close genetic affinity of Dwarf and KC germplasm has been reported in the current study and also in past studies (Perera et al., 2001; Dassanayaka et al., 2005; Dassanayaka et al., 2009). Interestingly, Perera et al. (2016) have hypothesized a common origin/domestication for Dwarf germplasm in Southeast Asia. A detailed molecular analysis of KC germplasm and Dwarf germplasm across South East Asia needed to be carried out in future to understand the detailed genetic relationships and origin/domestication of Kind Coconut germplasm.

This study was focused on the extensively distributed KC type; Thembili. However, there are four morphologically distinct KC types namely Thembili, Nawasi Thembili (Liyanage, 1958), Rathran Thembili (Wikramarathne, 1984) and Bothal Thembili available in Sri Lanka. Morphologically, Nawasi Thembili has an orange-colour epicarp and soft/edible mesocarp similar to Nawasi Tall. Rathran Thembili has a bright orange-colour epicarp, pink colour mesocarp and pink whorl under the perianth. Bothal Thembili has an orange colour epicarp and elongated unique nut shape (Ekanayake et. al., 2010). Nawasi Thembili, Rathran Thembili and Bothal Thembili are rare and valuable KC germplasm resources. Detailed studies on the genetic relationships of four KC types need to be carried out in future.

Furthermore, with the very high demand in the global market for KC nuts and nut water, there is an increasing demand for quality planting materials. However, there is no KC cultivar released or quality planting material available to date. This greatly limits the expansion of the beverage coconut industry in Sri Lanka. If detailed genetic studies and nut water quality analysis are carried out, that could facilitate to identification and release of a new KC cultivar for farmers in future, which could uplift the economic status of poor coconut growers which in turn improve the dying economy in Sri Lanka.

Conclusions

This study for the first time reports the existence of genetic diversity, population structure and genetic differentiation among indigenous King Coconut populations in Sri Lanka. A moderate level of genetic diversity (H_s=0.62), a clear genetic structure and moderately high (64%) genetic differentiations were reported for the selected KC populations. UPGMA phylogenetic tree clearly clustered five KC populations according to geographical locations: Pannala, Anuradhapura, Marandawila, Kadugannawa and Colambageara, Sri Lanka. Clear STRUCTURE was also observed for the KC populations. Detailed genetic studies with more KC populations especially from the total distribution range, genotyped with a higher number of SSR and/or SNP markers recommended before planning conservation and utilizing strategy for this valuable germplasm resource.

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Conflict of Interests

The authors declare no conflict of interest for this work.

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