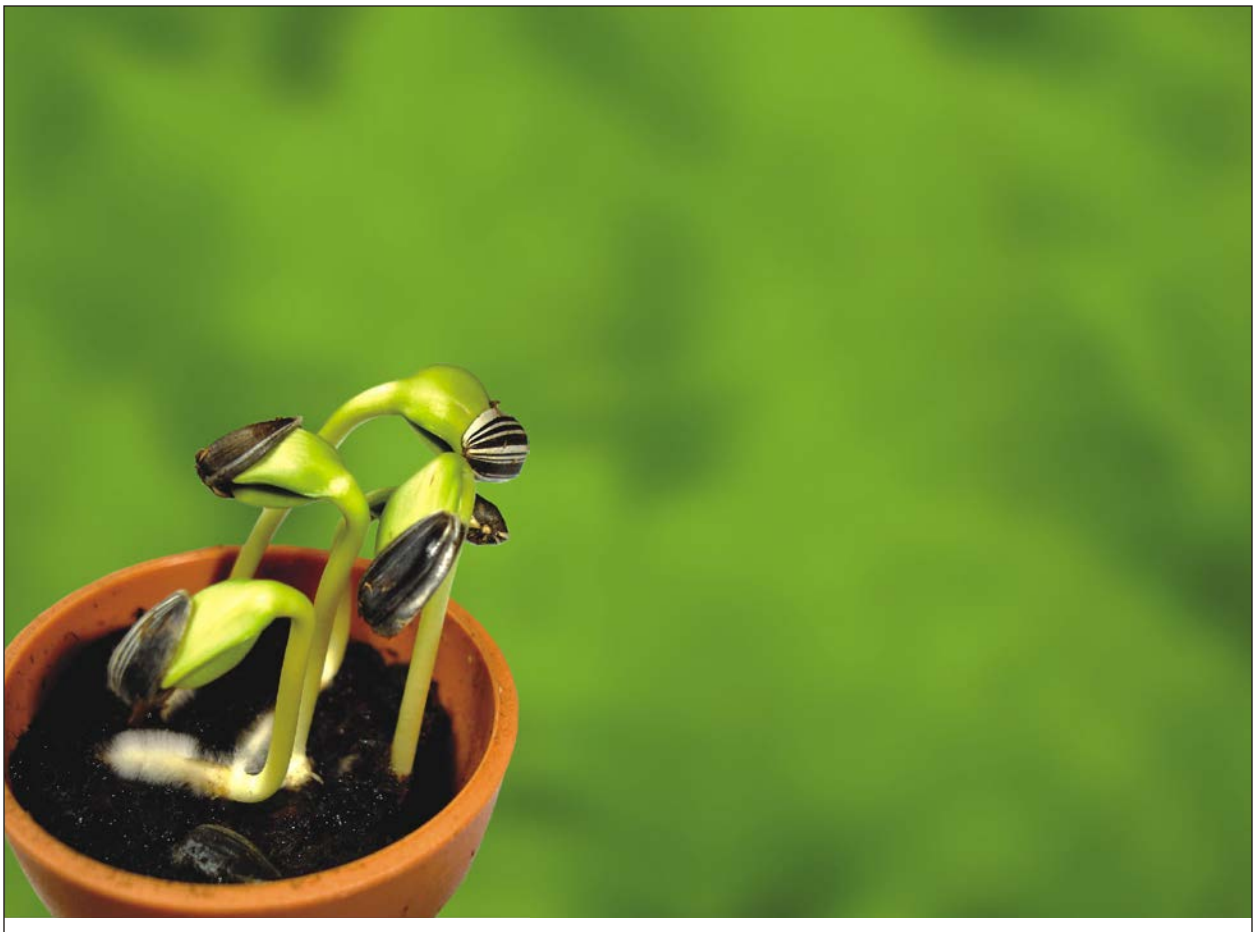

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Microsatellite Marker based Characterization of South Pacific Coconut (*Cocos nucifera* L.) Accessions

[1]S. Pradeep Kumar, [2]R. Manimekalai and [1]B.D. Ranjitha Kumari
[1]Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India
[2]Central Plantation Crops Research Institute, Department of Biotechnology, Kasaragod-21, Kerala, India

Corresponding Author: S. Pradeep Kumar, Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India Tel: 91-0431-2407061 Fax: 91-0431-2407045

ABSTRACT

The coconut palm (*Cocos nucifera* L.) is one of the major perennial oil crops of tropics providing the basis in many developing countries for food and industrial products. DNA based molecular marker techniques have proven powerful in genetic diversity estimation and it is advantages over morphological and biochemical markers. The aim of the present study was to identify the microsatellite primers (SSR) which are used to access genetic diversity among worldwide collection of germplasm (14 accessions). Assessment and distribution of genetic diversity was done with the help of software POPE GENE ver.1 and statistical parameters A total of 8 primers were used in the present study which produced 28 polymorphic alleles. The highest similarity index (0.7654) was observed between the accessions Hari papua Dwarf and kiriwana Tall the similarity coefficient ranged from 0.1775 to 0.7654 with a mean of 0.4231. The accessions grouped into three clusters in the dendrogram. Cluster 1 consisted of five accessions belonging to New Guinea. Cluster 2 consisted of four accessions belonging to French Polynesia. Cluster 3 consisted of five accessions belonging to Solomon Island were identified from south pacific coconut accessions.

Key words: DNA, coconut, pcr, microsatellite, pope gene

INTRODUCTION

Coconut belongs to the monotypic genus with a single species *Cocos nucifera*. It is presumed that the generic name *Cocos* as well as the popular name coconut are d Spanish word coco, meaning monkey face probably from the three scars on the base of the shell. This tree belongs to the family Arecaceae (Palmae) (Heatubun *et al*., 2009). Coconut palm is the tree of life. Each part of the coconut tree can be used to produce items of value for the community. Coconut palm is one of the major perennial oil crops of the tropics providing the basis in many developing countries for food products as well as serving industrial purposes (Jones, 1991; Persley, 1992). Coconut products provide food, shelter and energy to farm households and can be made into various commercial and industrial products. Fully developed and strategically used, it would increase food production, improve nutrition, create employment opportunities, enhance equity and help conserve the environment; hence it is called *Kalpavriksha* or tree of heaven.

Coconuts varieties can be classified as tall and dwarfs (Menon and Pandalay, 1958). Tall palms, sometimes referred as var. *typica,* were as Dwarf palms, sometimes referred as var. nana, are characterized by their short stature. Molecular markers provide an important technology for

evaluating levels and patterns of genetic diversity and have been utilized in a variety of plant species (Powell *et al.*, 1996; Rafalski *et al.*, 1996). Among the various DNA marker methods currently available 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 in 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). Microsatellite variability of coconut accessions (*Cocos nucifera* L.) from Andaman and Nicobar Islands (Rajesh *et al.*, 2008). Interrelationships among coconut (*Cocos nucifera* L.) accessions using RAPD technique (Manimekalai and Nagarajan, 2006). Genetic diversity and population genetic structure of salt marsh *Spartina alterniflora* from four coastal Louisiana basins (Utomo *et al.*, 2009). In this study we have utilized primers we previously designed to amplify polymorphic SSRs in coconut (Perera *et al.*, 1998) to study the levels and patterns of genetic diversity and population genetic structure of coconut palm obtained from various parts of the globe. Characterization of the genetic diversity of the tall coconut (*Cocos nucifera* L.) in the Dominican Republic using microsatellite (SSR) markers (Martinez *et al.*, 2010). The analysis of genetic diversity in coconut has been assessed for many years using morphological or biochemical traits (Meunier *et al.*, 1992; Fernando and Gajanayake, 1997). Characterization of coconut germplasm by microsatellite markers (Dasanayake *et al.*, 2003). Analysis of coconut (*Cocos nucifera* L.) diversity using microsatellite markers with emphasis on management and utilisation of genetic resources (Dasanayaka *et al.*, 2009). Diversity analysis of Central Asia and Caucasian lentil (Lens Culinaris Medik.) germplasm using SSR fingerprinting (Babayeva *et al.*, 2009). But this may not reliable measures because of genetic differences as influenced by environment on gene expression. In coconut, the molecular markers can be used to rationalize germplasm collections by reducing the duplications and maximizing the genetic diversity, to prioritize collection and conservation activities. Morphological markers (isozyme markers) and DNA based markers are used for the genetic diversity studies.

MATERIALS AND METHODS

The study was conducted at the Biotechnology Section, Crop Improvement Division of Central Plantation Crops Research Institute, Kasaragod, Kerala during 2005-2006. The plant materials for the molecular marker analysis consisted of spindle leaves of adult palms of world coconut germplasm center, obtained from coconut collections maintained at International Coconut Gene Bank for South Asia, Kidu, Dakshina Kannada district, Karnataka.

Collection of leaf samples: The following fourteen coconut accessions (3-5 palms per accession) conserved *ex situ* at International Coconut Gene Bank for South Asia, Kidu were selected for the present study. Details of Accessions code place of collection were shown in Table 1.

Isolation and quantification of plant genomic DNA: Total genomic DNA was extracted from one gram of spindle leaf tissue per coconut palm using standard protocol as described by Risterucci *et al.* (2000). The DNA was quantified spectrophotometrically and electrophoresing through 1.2% agarose gel checked the intactness.

SSR analysis: A total of 14 highly polymorphic SSR primer pairs from the coconut microsatellite kit (Baudouin and Lebrun, 2002) were used in the present study. PCR reaction was performed in

Table 1: Details of accessions code place of collection

Accessions	Code	Place
Bora tall	(BBT)	French polynesia
Haapiti tall	(HPT)	French polynesia
Hari papua dwarf	(HPD)	French polynesia
Rangiroa tall	(RGT)	French polynesia
Kaveing tall	(KVT)	New guinea
Nikkore dwarf	(ND)	New guinea
Tall muwa	(TM)	New guinea
Natava tall	(NVT)	New guinea
Niu ui tall	(NV)	New guinea
Kiriwana tall	(KWT)	Solomon island
Rennel tall	(RIT)	Solomon island
Solomon tall	(SIT)	Solomon island
Tutiala tall	(TUT)	Solomon island
Niu oma dwarf	(NOD)	Solomon island

Table 2: Details of SSR primers used in the analysis

Primer name	Forward primer (5'¬3')	Reverse primer (3'¬5')
CnCir B3	CATCTTGCTTTTCACCATCC	AATACTGTGCGGTTTTGCTT
CNZ 01	ATGATGATCTCTGGTTAGGCT	AAATGAGGGTTTGGAAGGATT
CNZ 02	CTCTTCCCATCATATACCAGC	ACTGGGGGGATCTTATCTCTG
CNZ 40	CTTGATTGCTATCTCAAATGG	CTGAGACCAAATACCATGTGT
CAC21	AATTGTGTGACACGTAGCC	GCATAACTCTTTCATAAGGA
CAC 23	TGAAAACAAAAGATAGATGTCA	GAAGATGCTTTGATATGGAAC
CAC71	ATAGCTCAAGTTGTTGCTAGG	ATATTGTCATGATTGAGCCTC
CAC84	TTGGTTTTTGTATGGAACTCT	A ACCAAAATGCTAACATCTCA

a 10 μL volume with a primer concentration of 0.2 μm each of forward reverse primer. Eight primer pairs were used to amplify the DNA (Table 2). Microsatellite loci were scored individually and the different alleles were recorded for each individual. The presence or absences of allele data were analyzed and using the software pope gene sizing of alleles was done by comparing the standard DNA. The allele size data were analyzed using the software pope gene.

Partitioning of genetic diversity: Partitioning of genetic diversity, within population and between populations was analyzed using G statistics. For each group of populations, the component of diversity within population was Hs/Ht and the component between populations was:

$$\text{Ht-Hs}\ \frac{\text{Gst}}{\text{Ht}}\ \text{(Nei, 1972)}$$

Where:
Ht : Total genetic diversity
Hs : Genetic diversity within population
Gst : Proportion of total diversity between populations

Gst is a measure of genetic variation between populations relative to that within populations. If Gst = 0, populations are not differentiated and diversity is not present within population and if Gst = 1 populations are highly differentiated and diversity is present between population.

Gene flow: Gene flow (Nm) was calculated for each group as:

$$Nm = 0.5 (1-Gst)/Gst$$

Gene flow destroys the diversity between populations. If Nm is more there is no difference between populations.

Polymorphism information content (PIC): The average polymorphism information content was calculated by applying the formulas given by Powell *et al.* (1996) and Smith (1997).

$$PIC = 1 - \sum_{i=1}^{n} fi^2,$$

where, fi is the frequency of the i the allele.

RESULTS

SSR analysis: Details of SSR markers produced by 8 coconut specific SSR primer pairs are furnished in Table 3 all the eight primer pairs produced unambiguous markers and were reproducible. The total number of alleles per primer pair ranged from three (CnCir B3, CAC 21, CAC 23, CAC 71, CAC 84) to five (CNZ 40). It produced a total of 28 markers across 14 coconut accessions. All the 28 markers were polymorphic; hence it produced 100% polymorphism. Averages of 3.5 alleles were observed per primer pair.

Allelic distribution of SSR marker: Among the 8 primer pairs used for SSR analysis, CNZ 40 gave the highest number of alleles (5). CnCir B3 produced the lowest number (3) of alleles. The allele distribution pattern is given in Table 4. The locus CNZ 01, CNZ02 and CNZ 40 had well distributed alleles among the coconut accessions. The locus CnCir B3, CAC 21, CAC23, CAC 71, CAC 84 had only three alleles with unequal distribution.

Polymorphism Information Content (PIC): PIC was calculated for SSR primers as per the formula. The primer CNZ 40 recorded the highest PIC value (0.8791) followed by CnCir B3 (0.4504). The lowest PIC value (0.0689) was recorded for CAC 23. The mean PIC value was 0.3795 (Table 5).

Table 3: Details of SSR markers produced with coconut germplasm accessions

Locus	Total alleles (No.)	Polymorphic alleles (No.)	Polymorphism (%)
CnCir B3	3	3	100
CNZ 01	4	4	100
CNZ 02	4	5	100
CNZ 40	5	5	100
CAC 21	3	3	100
CAC 23	3	3	100
CAC 71	3	3	100
CAC 84	3	3	100
Total	28	28	100
Mean	3.5	3.5	

Table 4: Allelic distribution of SSR markers among coconut germplasm accessions

Locus	Alleles total (No.)	No. of accessions for the Alleles					
		1	2	3	4	5	6
CnCir B3	3	21	25	28			
CNZ 01	4	9	14	3	13		
CNZ 02	4	26	18	20	19		
CNZ 40	5	20	29	28	31	1	
CAC 21	3	25	10	0			
CAC 23	3	25	15	22			
CAC 71	3	17	39	15			
CAC 84	3	29	29	23			

Table 5: Details of Polymorphism Information Content (PIC) among SSR primer pairs

Locus	Polymorphism information content (PIC)
CnCirB3	0.4504
CNZ 01	0.3278
CNZ 02	0.4308
CNZ 40	0.8791
CAC21	0.3966
CAC23	0.0689
CAC 71	0.2241
CAC 84	0.2586
Mean	0.3795

Table 6: Similarity matrix among coconut germplasm accessions based on SSR markers

	TM	NVT	KVT	KWT	ND	HPT	HPD	BBT	RGT	NV	RIT	SIT	TUT	NOD
TM	****													
NVT	0.3228	****												
KVT	0.2404	0.2483	****											
KWT	0.7534	0.4551	0.6086	****										
ND	0.3550	0.2585	0.1801	0.6408	****									
HPT	0.2030	0.2099	0.2512	0.6020	0.2668	****								
HPD	0.6740	0.4802	0.7654	0.3650	0.4183	0.4504	****							
BBT	0.3569	0.2590	0.3129	0.6152	0.4061	0.4095	0.6944	****						
RGT	0.5265	0.3444	0.4476	0.5752	0.4525	0.3379	0.6663	0.4389	****					
NV	0.3292	0.3843	0.3001	0.7295	0.3296	0.2591	0.4759	0.3959	0.5344	****				
RIT	0.4883	0.2963	0.2607	0.7215	0.3322	0.3621	0.7023	0.5299	0.5049	0.2721	****			
SIT	0.3780	0.1839	0.2916	0.5124	0.2967	0.2860	0.5626	0.4230	0.3721	0.3022	0.1775	***		
TUT	0.4242	0.2883	0.3171	0.5475	0.2875	0.3492	0.5091	0.5134	0.6721	0.3281	0.2460	0.908	****	
NOD	0.4846	0.6275	0.4883	0.6587	0.4703	0.3741	0.4020	0.4550	0.4618	0.4516	0.4606	0.4851	0.422	****

Cluster analysis / similarity information index: The similarity index obtained for each pair wise comparison among the 14 coconut accessions was presented in Table 6. The highest similarity index (0.7654) was observed between the accessions HPD and KWT. The similarity coefficient ranged from 0.1775 to 0.7654 with a mean of 0.4231. The similarity coefficient O.1775 indicated the absence of similarity between accessions.

Cluster I consisted of five accessions belonging to New Guinea (5).Cluster II consisted of four accessions belonging to French Polynesia. Cluster III consisted of five accessions belonging to Solomon Island.

Table 7: Distribution of coconut germplasm accessions in the phonetic tree classified by geographic origin based on SSR markers

Cluster	NG	FP	SI	Total
1	3	1	1	5
2	1	*	3	4
3	*	2	1	3
4	1	1	*	2
Total	5	4	5	14

*Denotes absent in the cluster of phonetic tree. NG: New Guinea, FP: French Polynesia, SI: Solomon Island

Population genetic structure analysis: The SSR primers detected total 28 markers across 14 coconut accessions and all were polymorphic. The binary data were analyzed using the software POPGENE' ver 1.32 (Yeh *et al.*, 1999; Raymond and Rousset, 1995). The results were furnished in Table 6. The number of observed alleles among populations varied from 1.0000 (KWT) to 1.8800 (NVT). The number of effective alleles ranged from 1.0000 (KWT) to 1.5271 (NVT). Gene diversity for each population varied from 0.0000 (KWT) to 0.3131 (NVT). Among the 14 populations, NVT produced the highest number of polymorphic marker (22).

Shannon's index and multiple population analysis: Shannon's index provided information regarding within population diversity. The Shannon's index for individual population was given in Table 8. The population NVT had the highest index while the population KWT had the lowest index. Among the three groups, the population belonging to the group II (French Polynesia) produced the highest number of observed allele (1.9643), the highest number of effective alleles (1.6499), the highest gene diversity (0.3767) (Table 7).

Population genetic identity/genetic distance and clustering: Of the three groups, the mean genetic identity was the highest for the Group III (0.34428) followed by Group I (0.17915). Group II has the lowest mean genetic identity (0.0172). The dendrogram based on Nei's genetic distance of 14 accessions showed 3 clusters (Fig. 1). The list of three clusters along with the accessions included is given in (Table 8). Cluster I consisted of five accessions belonging to New Guinea (4) and French Polynesia (1). Cluster II consisted of five accessions belonging to Solomon Islands (4) and French Polynesia (1). Cluster III consisted of New Guinea (1), French Polynesia (2) and Solomon Islands (1).

Partitioning of genetic diversity: Partitioning of genetic diversity in to within population and between populations was calculated for each group of population using Gst. The total diversity (Ht) was the highest (0.3131) for the Group III. The 'with in population' diversity was high (0.1919) for the group I. The proportion of total diversity present with in population was high for the Group II (0.9937) Gst measured of genetic variation between population relative to that with in population. If Gst = 1 population was highly differentiated and diversity was present between population. Highest 'with in population' diversity was present in Group I (Gst = 0.62%). On a average high between population was present in Group III (40.79%) Gene flow (Nm) was calculated for each group of population. If Nm is more, there is no difference between the populations. Group I had high Nm (80.1711) and hence there was no difference between the population with that Group on the other hand the Group III had less Nm and hence their population are differentiated each other. On an average with in population diversity was high (0.8478%) than between population diversity (0.1520%) (Table 9).

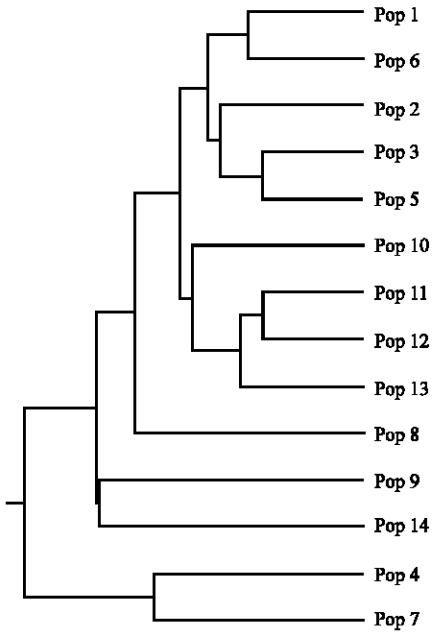

Fig. 1: Dendrogram of coconut molecular marker analysis based on SSR markers. Pop 1: TM, Pop2: NVT, Pop 3: KVT, Pop 4: KWT, Pop 4: ND, Pop 6: HPT, Pop 7: HPD, Pop 8: BBT, Pop 9: RGT, Pop 10: NV, Pop 11: RIT, Pop 12: SIT, Pop 13: TUT, Pop 14: NOD

Table 8: Details of variability parameters generated among coconut populations base SSR markers

Population name	Observed No. of alleles (n)	Effective No. of alleles (ne)	Nei's gene diversity (h)	Shannon's information index (I)	No. of polymorphic loci	Percentage of polymorphic loci
TM	1.5714	1.3783	0.2160	0.3191	16	57.14
NVT	1.8800	1.5271	0.3131	0.4701	22	78.57
ND	1.6786	1.4318	0.2501	0.3719	19	67.86
KVT	1.0556	1.0393	0.0230	0.0336	1	3.57
NV	1.5000	1.3525	0.1989	0.2912	14	50.00
HPD	1.6786	1.5005	0.2770	0.4021	19	67.86
KWT	1.0000	1.0000	0.0000	0.0000	0	0.00
HPT	1.5000	1.3190	0.1870	0.2781	14	50.00
BBT	1.2800	1.2249	0.1216	0.1744	7	25.00
RIT	1.5926	1.3807	0.2212	0.3284	16	57.14
TUT	1.5000	1.3405	0.1936	0.2848	14	50.00
SIT	1.6429	1.3826	0.2228	0.3353	18	64.29
RGT	1.5000	1.3020	0.1778	0.2666	14	50.00
NOD	1.3600	1.2197	0.1337	0.2003	9	32.14

Table 9: Details of genetic variation present between population and within populations based on SSR markers

Group	Sample size (No.)	Ht	Hs	Hs /Ht	Gst	Nm
1	21	0.1931	0.1919	0.9937	0.0062	80.1711
2	14	0.1494	0.1431	0.9578	0.0420	11.4161
3	23	0.3131	0.1854	0.5921	0.4079	0.7258
Mean				0.8478	0.1520	
Average of groups I and III				0.7929	0.2070	

DISCUSSION

In the past the analysis of genetic diversity in coconut has been assessed using morphological or biochemical traits (Meunier *et al.*, 1992; Fernando and Gajanayake, 1997). But this may not be a reliable measure because of genetic differences as influenced by environment on gene expression. Studying diversity using leaf polyphenol polymorphism provided variability; however, the sensitivity of the polyphenol banding patterns to geographical variations limits its use (Jay *et al.*, 1989). Molecular markers have alleviated scope of these difficulties associated with germplasm analysis by making it possible to collect samples at remote sites and analyses there. In the present investigation SSR markers were used for the estimation of genetic diversity among the coconut germplasm accessions.

Population genetic structure analysis reveals distribution of alleles and change in allele frequencies under their influence of the evolutionary forces and it helps to observe the distribution of diversity within and between populations. In the present study SSR markers were used for the estimation of genetic diversity among the 14 coconut germplasm accessions (48 individuals) were used. SSR markers produced high level of polymorphism and were generated three to five alleles with an average of 4.166 alleles/locus. Earlier 7.4 alleles/locus (Perera, 2003), 4.4 alleles/locus (Dasanayake *et al.*, 2003) and 5.2 alleles/locus (Rivera *et al.*,1999) were reported.

In the present investigation, polymorphism information content (PIC) was calculated for SSR primers. PIC provides an estimate of the discriminating power of a marker. SSR markers showed a mean PIC value of 0.3785. Shannon index revealed the genetic diversity within population. Among the coconut populations studied KWT (0.4701) had the highest index while the population NV (0.000) had the lowest index. To distribute the genetic diversity and to reveal variation within and among groups based on geographical region and plant structure, the coconut populations classified into 3 groups.

The group containing Little Andaman accession showed reduced number of polymorphic alleles and within population diversity. In the present study it was found that the total genetic diversity was partitioned more in within population (64.48 %) rather than between populations (35.52 %). When a Gst approaches I, which indicates populations are highly differentiated and diversity is present 'between populations'. In the present study, Nicobar Islands coconut populations exhibited relatively higher between population diversity (Gst = 0.5758) and the little Andaman coconut populations exhibited lower between population diversity (Gst = 0.0062). These results also suggest the importance of prior knowledge on the amount and distribution of genetic diversity among population for appropriate collection and conservation strategies in coconut. Earlier germplasm collection has been on morphology and quantitative data. The criterion for germplasm collection based on the in situ measurement (growth habit and fruit component data) alone was not a useful measurement of genetic diversity as the environment had large influence on them. To evaluate genetic diversity more exactly, we are increasing number of DNA samples and kinds of SSRs to be examined. Comparison of morphological and genetic diversity is being undertaken. The molecular data generated in the present study will help to formulate accurate collection strategies. Individual strategies must be considered for different regions on the level and distribution of the genetic diversity in the particular area.

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