SSR and ISSR Markers based Population Genetic Structure of Coconut (*Cocos nucifera* L.) Germplasm Accessions

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The coconut palm (*Cocos nucifera* L.) is one of the major perennial oil crops of tropics. Population genetic structure was assessed among 33 accessions (4 individuals per accession) with ISSR and SSR markers. The molecular marker data were analyzed with POPGENE and ARLEQUIN software. The parameters derived were, Shannon's index, differentiation indices (Fst and Gst) and molecular variances. The diversity was partitioned into 'within population' (59.82% and 68.56% based on ISSR and SSR markers, respectively) and 'between populations' (40.18% and 31.44% based on ISSR and SSR markers, respectively). Relatively high 'between populations' diversity was present in the accessions belonging to South Pacific region reflecting higher population differentiation. Dwarfs and intermediate coconut types also maintained high 'between populations' diversity due its autogamous behaviour. There was overall reduction in the number of markers (ISSR and SSR) among the dwarfs and intermediate populations. The study provided useful information regarding the genetic makeup of the coconut germplasm accessions and their utilization in breeding.

Key Words: Coconut, Diversity, ISSR, Population structure, SSR

Introduction

Coconut (*Cocos nucifera* Linn.) is named as Tree of Abundance, Tree of Life, the Consols of East etc., is an important perennial oil yielding plantation crop of the tropics. *C. nucifera* (*2n = 2 x* = 32) is a member of monocotyledonous family Arecaceae (Palmaceae). It is the only species of the genus *Cocos* belonging to the subfamily Cocoideae. Almost every part of the tree is used for its food and industrial products. It has been grown in 86 countries including India. Presently, the crop is now facing relative decline in cultivation in many countries, largely due to the impact of diseases on the yields and low farmer productivity. Hence, an urgent need is to evolve high yielding coconut palms either through hybridization / through selection in well collected and maintained germplasm. Assessment of population genetic structure will help in germplasm collecting, conservation and utilization processes.

Various molecular marker techniques like RFLPs (Lebrun *et al*., 1998), RAPD (Ashburner *et al*., 1997; Everard, 1999; Upadhyay *et al*., 2004), AFLP (Perera *et al*., 1998; Teulat *et al*., 2000), ISTR (Rohde *et al*., 1995), SSR (Perera *et al*., 1999; Rivera *et al*., 1999, Meerow *et al*., 2003) and ISSR have been reported in coconut.

In the present study, population genetic structure among world wide collections of coconut germplasm accessions was assessed using DNA markers, *viz*., ISSR and SSR.

Materials and Methods

One hundred and thirty two individual palms from 33 coconut germplasm accessions (4 palms per accession referred as population) were used. The accessions belong to a collection maintained at International Coconut Gene Bank for South Asia. These are conserved *ex situ* at Central Plantation Crops Research Institute, Kasaragod, India. These accessions belong to different geographic origin. The details on the plant stature (type), origin (place of collection) and geographic location are available Manimekalai and Nagarajan (2006). The accessions were grouped according to the geographic region as accessions belonging to South East Asia, South Pacific, South Asia, Atlantic and America and Africa.

DNA Extraction

DNA was extracted using Plant DNA extraction kit (Invitrogen) as per the manufacturer's instructions.

ISSR Analysis

Primers targeting the SSR were obtained from University of British Columbia (Canada). Amplification reactions were carried out in 10 ml volume containing 30 ng of template DNA, 200 mM of each dNTPs, 0.45 U of Taq polymerase (Bangalore Genei Pvt. Ltd. India)

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and 0.8 mM of primer. The PCR products were subjected to electrophoresis through a 1.80% agarose gel using 1X TBE buffer at 90 volts for 3 h in Bio-Rad submarine electrophoresis unit. The ethidium bromide stained gels were documented using the Alpha Imager^{TM} 1200 Documentation and Analysis system (Alpha Innotech Corporation, USA).

SSR Analysis

SSR analysis was carried out as described by Perera *et al.* (2000). Primer sequences were obtained from CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement), Montpellier, France).

Data Analysis

ISSR markers

Only the clear, unambiguous and reproducible bands were considered for scoring. Each band was considered to be a single locus. Data were scored as "1" for the presence and "0" for the absence of a DNA band of each accession. DNA band size was estimated by comparing the DNA bands with a 1 Kb DNA ladder. The binary data matrix was entered into the software POPGENE version 1.32 (Yeh and Boyle, 1999). The population genetic diversity parameters estimated were, observed for number of alleles per locus, effective number of alleles per locus, Nei's (1973) gene diversity, Shannon's information index and G statistics. For describing coconut population based on their geographic region and plant type, 6 groups have been identified. Group I consisted of tall populations of South East Asia; Group II comprised of South Pacific populations; Group III consisted of Atlantic and American populations; Group IV has populations belonging to Africa; Group V consisted of South Asian populations. The dwarfs and intermediate types are put under Group VI.

SSR markers

Microsatellite loci were scored individually and the different alleles were recorded for each population. Sizing of alleles was done by comparing with 30 bp ladder.

Table 1. Details of coconut germplasm populations, place of collection and geographic region

No.	Accession	Type	Country of origin	Geographic region
1	Kong Thienyong Tall	Tall	Borneo	Southeast Asia
2	Straight Settlement Green Tall	Tall	Malaysia	Southeast Asia
3	Straight Settlement Apricot Tall	Tall	Malaysia	Southeast Asia
4	Philippines Kalambahim Tall	Tall	Philippines	Southeast Asia
5	Laguna Tall	Tall	Philippines	Southeast Asia
6	Philippines Palawan Tall	Tall	Philippines	Southeast Asia
7	Philippines Dalig Tall	Tall	Philippines	Southeast Asia
8	San Roman Tall	Tall	Philippines	Southeast Asia
9	Markham Valley Tall	Tall	Papua New Guinea	South Pacific
10	Nufella Tall	Tall	New Caledonia	South Pacific
11	Nugili Tall	Tall	New Caledonia	South Pacific
12	Nuwallis Tall	Tall	New Caledonia	South Pacific
13	Nu Quamen Tall	Tall	New Caledonia	South Pacific
14	Kupien Tall	Tall	New Caledonia	South Pacific
15	Nuwehnug Tall	Tall	New Caledonia	South Pacific
16	Lifou Tall	Tall	Guam Islands	South Pacific
17	British Solomon Island Tall	Tall	Solomon Islands	South Pacific
18	Jamaica Tall	Tall	Jamaica	Atlantic
19	Saint Vincent Tall	Tall	Trinidad	Atlantic
20	Panama Tall	Tall	Panama	America
21	Nigerian Tall	Tall	Nigeria	Africa
22	Kaithathali Tall	Tall	India	South Asia
23	Indian Spicata	Tall	India	South Asia
24	Indian East Coast Tall	Tall	India	South Asia
25	Verrikobbari Tall	Tall	India	South Asia
26	Nadora Tall	Tall	India	South Asia
27	Nicobar Tall	Tall	India	South Asia
28	Hazari Tall	Tall	India	South Asia
29	Navassi Tall	Tall	India	South Asia
30	Niuleka Dwarf	Intermediate	Fiji	South Pacific
31	King coconut	Intermediate	Sri Lanka	South Asia
32	Laccadive Dwarf	Dwarf	India	South Asia
33	Chowghat Orange Dwarf	Dwarf	India	South Asia

(Ratnambal *et al*., 1995; 2000)

The allele size data were analyzed using the software ARLEQUIN version 2.0 (Schneider *et al*., 2000). For each population the genetic diversity parameters estimated were number of polymorphic sites and gene diversity. The genetic structure of population was investigated by Analysis of Molecular Variance (AMOVA) (Excoffier *et al.,* 1992).

Results

Based on ISSR Markers

Variability and Level of Polymorphism

ISSR primers detected a total of 120 markers across 33 coconut populations, out of which 104 were polymorphic (86.6 %). The number of markers for each primer varied from nine (UBC 854) to 17 (UBC 854), with a mean of 12 markers per primer. The number of polymorphic markers for each primer varied from seven (UBC 835) to 15 (UBC 854 and UBC 855) with a mean of 10.4. The product size ranged from 206 bp to 2618 bP (Table 2).

The number of observed alleles among populations varied from 1.1667 (King coconut) to 1.7833 (Nicobar tall). The number of effective alleles ranged from 1.1404 (King coconut) to 1.6833 (Nicobar tall). Gene diversity for each population varied from 0.0746 (King coconut) to 0.3555 (Nicobar Tall). Among 33 coconut populations, Nicobar tall produced the highest number of polymorphic markers (94), while King coconut had the least (20).

Shannon's Information Index

Shannon's index provided information regarding within population diversity. The Shannon's index for individual population is given in Table 3. The population, Nadora tall had the highest index (0.5032). While, King coconut had the lowest index (0.1061) followed by Philippines Palawan tall (0.1615), Nuwallis tall (0.1702) and Straight Settlement Apricot tall (0.1760). The mean Shannon's index was 0.2687. The mean Shannon's index among the tall populations was higher (0.2823) when compared to the dwarf and intermediate populations (0.2323).

Multiple Population Analysis

Partitioning of genetic diversity in to 'within population' and 'between populations' was calculated for each group of populations using Gst. The total diversity (Ht) was the highest (0.3652) for the populations belonging to South Asia (Group V) and the 'within population' diversity was also high for that group (0.2256). The proportion of total diversity present 'within population' was high for the Atlantic and American accessions (Group III) (0.6443).

Table 2. Details of ISSR markers produced among coconut populations

Primer	Total markers (No.)	Polymorphic markers (No.)	Polymorphism (%)	Product size (bp)
UBC815	13	13	100.0	2618-554
UBC834	12	10	83.3	1252-206
UBC841	10	8	80.0	2316-698
UBC810	9	8	88.8	2443-879
UBC824	10	9	90.0	2455-506
UBC835	10	7	70.0	2375-514
UBC854	17	15	88.2	1545-299
UBC855	15	15	100.0	2459-606
UBC889	14	10	71.4	2069-290
UBC823	10	9	90.0	2287-877
Total	120	104	86.6	
Mean	12.0	10.4	86.6	

Table 3. Shannon's index based on ISSR markers for the individual coconut population

Population	Shannon's index
Kong Thienyong Tall	0.2673
Straight Settlement Green Tall	0.2439
Straight Settlement Apricot Tall	0.1760
Philippines Kalambahim Tall	0.2342
Laguna Tall	0.2350
Philippines Palawan Tall	0.1615
Philippines Dalig Tall	0.2990
San Roman Tall	0.1890
Markham Valley Tall	0.2241
Nufella Tall	0.2306
Nugili Tall	0.3948
Nuwallis Tall	0.1702
Nu Quamen Tall	0.2428
Kupien Tall	0.3821
Nuwehnug Tall	0.2294
Lifou Tall	0.3255
British Solomon Island Tall	0.3648
Jamaica Tall	0.2626
Saint Vincent Tall	0.3005
Panama Tall	0.2699
Nigerian Tall	0.4336
Kaithathali Tall	0.2259
Indian Spicata	0.2850
Indian East Coast Tall	0.4501
Verrikobbari Tall	0.2392
Nadora Tall	0.5032
Nicobar Tall	0.2329
Hazari Tall	0.2372
Navassi Tall	0.2169
Niuleka Dwarf	0.2420
King coconut	0.1061
Laccadive Dwarf	0.2146
Chowghat Orange Dwarf	0.2757
Mean	0.2687
Mean among the talls	0.2823
Mean among the dwarfs	0.2323

Highest 'within population' diversity was present among Atlantic and American accessions (Group III , Gst = 35.57%) followed by South Asian accessions (Group V, Gst = 38.21%). Relatively high 'between population' diversity was present among South Pacific accessions (Group II , Gst = 42.98%) and Intermediate and dwarf types (Group VI, 42.92%). On an average 'within population' diversity was higher (59.82%) than 'between population' diversity (40.18%) (Table 4).

Based on SSR Markers

Variability and Level of Polymorphism

Seven highly polymorphic SSR loci detected a total of 82 alleles, and all were polymorphic. The total number of alleles for each locus varied from two (CnCirE12) to 17 (CnCirE2) with an average of 11.71 alleles per locus. The allele size varied from 115 bp (CnCirE2) to 278 bp (CnCirE10) (Table 5). The number of polymorphic sites (Polymorphic alleles) ranged from 0 to 7. The intermediate population, King coconut had no polymorphic sites. The dwarf population, Chowghat Orange Dwarf had only 2 polymorphic sites. The intermediate population Niuleka Dwarf, had more polymorphic sites (6) when compared to other intermediate population King coconut (0). Among tall populations, Navassi Tall had the least number of polymorphic sites (3). The average number of polymorphic sites was calculated and found to be more among the tall populations (6.45) compared to dwarf populations (3.50). Total number of alleles was more among the tall populations (82) when compared to dwarfs (17). Gene diversity among 33 coconut populations varied from 0.734 (Nugili tall) to 0.000 (King coconut).

Population Genetic Structure Inferred by Analysis of Molecular Variance (AMOVA)

The partitioning of diversity into 'among groups' of coconut population, 'between populations' and 'within population' is shown in Table 6. The diversity present 'among the groups' was highly significant (13.10 %) reflecting a moderate differentiation among the geographical groups of coconut populations. The diversity present 'between population' was 18.38 per cent, which was highly significant reflecting great differentiation among populations. The component 'within population' accounted 68.56 per cent of diversity and found to be highly significant. The population differentiation was also reflected by fixation indices. The Fst statistic was 0.31437, which was found to be highly significant reflecting great level of diversity present 'between populations' and 'between groups'. Accordingly the within population diversity accounted for 68.56% of observed diversity in the coconut germplasm used in this study. On an average, the total diversity was partitioned 'within population' (68.56%) rather than between populations (31.44%).

Discussion

Based on ISSR Markers

ISSR primers have exhibited high per cent (86.6) of polymorphism reflecting its high informativeness. In the present study, it was found there was reduction in number of alleles and effective alleles in dwarfs and intermediate populations (King coconut, Laccadive dwarf and Chowghat orange dwarf). This result was in agreement with report of Perera *et al.* (2003).

Based on Shannon's index, Nadora tall showed the highest genetic diversity within it. The least genetic diversity was shown by King coconut. The mean Shannon index among talls was found to be higher (0.2823) when compared to the dwarfs and intermediates (0.2323). Among the talls, Straight settlement apricot tall, Philippines palawan tall, San ramon tall and Nuwallis tall were found to have less index and consequently less 'within population' diversity. The diversity was for the South asian population was contributed by Nicobar tall, Verrikobbari tall and Kaithathali tall. Previously, Upadhyay *et al.* (2004) reported higher diversity in the Philippine

Table 4. Partitioning of genetic diversity between populations and within population based on ISSR markers

Group	Individuals (No.)	Ht	Hs	Hs / Ht	Gst	Nm
I	32	0.2645	0.1554	0.5874	0.4126	0.7119
II	36	0.3159	0.1801	0.5702	0.4298	0.6633
III	12	0.3373	0.2173	0.6443	0.3557	0.9058
V	32	0.3652	0.2256	0.6179	0.3821	0.8085
VI	16	0.2546	0.1453	0.5708	0.4292	0.6650
Mean				0.5982	0.4018	

Group IV excluded from the analysis because of single population ; Ht - Total genetic diversity; Hs - Within population genetic diversity; Hs/Ht- Proportion of total genetic diversity "within population"; Gst - Proportion of total genetic diversity "between population"; Nm- Gene flow

Table 5. Total alleles and product size of the SSR markers produced across coconut populations

Locus	Total alleles (No.)	Product size (bp)
CnCirA3	9	210-248
CnCirB12	15	135-183
CnCirC3	18	188-272
CnCirC12	10	120-190
CnCirE2	17	115-185
CnCirE10	11	226-278
CnCirE12	2	164-174
Total	82	
Mean	11.71	

Table 6. Analysis of molecular variance based on average of seven SSR loci

Source of variation	Sum of squares	Variance components	Diversity (%)
Among groups	109.151	0.37215 Va	13.10**
Between populations	165.857	0.52043 Vb	18.33**
Within population	453.575	1.94667 Vc	68.56**
Total	728.583	2.83926	

Fst : 0.31437; va- variance components among groups; vb-variance components between population; vc-variance components within population

Ordinary Talls based on Shannon's index. Shanon's index reflect the within population diversity (Ashburner *et al*., 1997). Dwarfs and intermediate populations showed reduced number of polymorphic alleles and 'within population' diversity. Reduction in 'within population' diversity in dwarfs and intermediate types are also reported (Perera *et al*., 1998; Upadhyay *et al*., 2004).

Based on ISSR markers, genetic diversity was partitioned more in 'within population' (59.82%) rather than 'between population' (40.18%). This observation was in accordance with the earlier studies using RAPD (Upadhyay *et al*., 2004), SSRs (Perera *et al*., 2001). Gst values which reflect the population differentiation was least for the Atlantic and American populations which revealed high 'within population' diversity. Earlier Ashburner *et al.* (1997) analyzed a set of South Pacific coconut populations and reported more of 'within population' diversity (60%) and proportion of the diversity found between populations was less (40%). Among the five different geographic groups, relatively higher 'between population' diversity was present in the populations of South Pacific region (Gst = 0.4298). It was suggested that relatively high 'between population' diversity in the coconut populations of the South Pacific region has probably arisen because of the establishment of populations by few individuals (Ashburner *et al*., 1997). The low differentiation of populations of South Asia and Atlantic and America may be attributed to the gene flow between populations. High gene flow between populations will reflect low levels of population differentiation. However very few individuals were used for the present study and few for Atlantic and American accessions.

Based on SSR Markers

Seven SSR loci detected 100% polymorphism among 33 coconut populations. One of the dwarf populations namely, Chowghat orange found to have 71% of homozygous loci. The intermediate populations, King coconut showed homozygous at all 7 loci. Niu Leka dwarf , known to be a cross–pollinator among the dwarfs was homozygous for only one locus out of 7 loci tested. Since it is known to be a cross-pollinator. In general, dwarf populations showed a reduction in diversity and in number of total alleles (17) when compared to talls (82). This result agrees with Perera *et al.* (2003) who suggested that the dwarf coconuts are subset of tall coconuts and was directly evolved from tall coconut variety as a result of an event of domestication.

The hierarchical analysis of molecular variance (AMOVA) partitioned most of the diversity (68.56%) 'within population'. This result was comparable with the result obtained by ISSR markers in the present study. AMOVA suggested moderate level of differentiation between groups based on geographic region and plant stature. Higher 'within population' diversity obtained in the present study agrees with the general observation that woody perennial out breeding species like coconut and other crops maintain most of their variation within population (Hamrick and Godt, 1989; Bartish *et al*., 2000; Perera *et al*., 2001; Oraguzie *et al.*, 2001; Archak *et al*., 2003; Belaj *et al*., 2003).

The results obtained based on ISSR and SSR markers were comparable, however, SSR markers were more efficient in describing homozygous / heterozygous nature of the population. The data obtained suggest the importance of prior knowledge on the amount and distribution of genetic diversity among population for appropriate collection and conservation strategies. Diverse accessions from different geographic regions could be utilized in heterosis breeding.

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References

Archak S, AB Gaikwad, D Gautam, EVVB Rao, KRM Swamy and JL Karihaloo (2003) DNA fingerprinting of Indian cashew (*Anacardium occidentatle* L.) varieties using RAPD and ISSR techniques. *Euphytica* **230:** 397-404.

Ashburner GR, WK Thompson and GM Halloran (1997) RAPD analysis of South Pacific coconut palm populations. *Crop Sci.* **37:** 992-997.

Bartish IV, LP Grakava, K Rumpunen and H Nybom (2000) Phylogenetic relationships and differentiation among and within populations of *Chaenomeles* Lindl. (Rosaceae) estimated with RAPDs and isozymes. *Theor. Appl. Genet.***101:** 554-563.

Belaj A, Z Satovic, H Ismaili, D Panajoti, L Rallo and I Trujillo (2003) RAPD genetic diversity of Albanian olive germplasm and its relationships with other Mediterranean countries. *Euphytica* **130:** 387-395.

Everard JMDT (1999) An investigation towards developing a molecular approach to improve the efficiency of coconut breeding by RAPD marker assisted selection. *CORD* **15:** 115-130.

Excoffier L, P Smouse and J Quattro (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479-491.

Hamrick L and MJW Godt (1989) Allozyme diversity in plant species. In: AHD, Brown, MT Clegg, AL Kahler and BS. Weir (Eds) *Plant Population Genetics, Breeding and Genetic Resources*, Sinauer, Sunderland, pp 43-63.

Lebrun P, YP N'Cho, M Seguin, L Grivet and L Baudouin (1998) Genetic diversity in coconut (*Cocos nucifera* L.) revealed by restriction fragment length polymorphism (RFLP) markers. *Euphytica* **101:** 103-108.

Manimekalai R and P Nagarajan (2006) Inter-relationship among coconut (*Cocos nucifera* L.) germplasm accessions using RAPD technique *Genet. Resour. Crop Evol.* **53:** 1137-1144.

Meerow AW, RL Wisser, JS Brown, DN Kuhn, RJ Schnell and Broschat TK (2003) Analysis of genetic diversity and population structure within Florida coconut (*Cocos nucifera* L.) using microsatellite DNA with special emphasis on the Fiji Dwarf cultivar. *Theor. Appl. Genet.* **106:** 715-726.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci.* **70:** 3321-3323.

Oraguzie NC, SE Gardiner, CM Basset, M Stefanati, RD Ball, VGM Bus and AG White (2001) Genetic diversity and relationships in *Malus* sp. germplasm collections as determined by randomly amplified polymorphic DNA. *J. Amer. Soc. Hort. Sci.* **126:** 318-328.

Perera L, JR Russell, J Provan and W Powell (1999) Identification and characterization of microsatellites in coconut (*Cocos nucifera* L.) and the analysis of coconut population in Sri Lanka. *Mol Ecol.* **8:** 344-346.

Perera L, JR Russell, J Provan and W Powell (2000) Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (*Cocos nucifera* L.) *Genome* **43:** 15-21.

Perera L, JR Russell, J Provan and W Powell (2001) Levels and distribution of genetic diversity of coconut (*Cocos nucifera* L. var. *Typica* form *typica*) from Sri Lanka assessed by microsatellite markers. *Euphytica* **122:** 381-389.

Perera L, JR Russell, J Provan and W Powell (2003) Studying genetic relationships among coconut varieties/populations using microsatellite markers. *Euphytica* **132:** 121-128.

Perera L, JR Russell, J Provan, JW McNicol and W Powell (1998) Evaluating genetic relationships between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. *Theor. Appl. Genet.* **96:** 545-550.

Rivera R, KJ Edwards, JHA Barker, GM Arnold, G Ayad, T Hodgkin and A Karp (1999) Isolation and characterization of polymorphic microsatellites in *Cocos nucifera* L. *Genome* **42:** 668-675.

Rohde W, A Kullaya, J Rodriguez and E Ritter (1995) Genome analysis of *Cocos nucifera* L. by PCR amplification of spacer sequences separating a subset of copia-like *Eco*RI repetitive elements. *J. Genet. Breed.* **49:** 179-186.

Schneider S, D Roessli and L Excoffier (2000):ARLEQUIN 2.0. A software for population genetic data analysis. University of Geneva, Switzerland.

Teulat B, C Aldam, R Trehin, P Lebrun, JHA Barker, GM Arnold, A Karp, L Baudouin and F Rognon (2000) An analysis of genetic diversity in coconut (*Cocos nucifera*) population from across the geographical range using sequence tagged microsatellites (SSRs) and AFLPs. *Theor. Appl. Genet.* **100:** 764-771.

Upadhyay A, K Jayadev, R Manimekalai and VA Parthasarathy (2004) Genetic relationship and diversity in Indian coconut accessions based on RAPD markers. *Scientia Hort.* **99:** 353-362.

Yeh FC and T Boyle (1999) POPGENE VERSION 131 a Microsoft Window-based software for population genetic analysis University of Alberta, Canada.