Original Research Article

Genetic Variation among Varieties, Wild Species and Related Genera of Arecanut (*Areca catechu* L.) as Revealed by Microsatellite Markers

Abstract

Studies on genetic diversity of crops are crucial for germplasm conservation and crop improvement programmes.Arecanut (*Areca catechu* L.) is one of the plantation crops that have been in cultivation since time immemorial in tropical regions of the world. In this investigation, molecular characterization and genetic diversity analysis was carried out among 20 arecanut genotypes comprising of 16 cultivated types (including 13 cultivated varieties, two dwarf hybrids and a natural mutant), two wild species (*Areca triandra*and *Areca concinna)* and two other genera *(Normanbyanormanbyii*and*Actinorhytiscalapparia),* maintained in the National Arecanut Gene Bank, Vittal, Karnataka State, India, by employing eight simple sequence repeat (SSR) markers. A total of 45 alleles were detected with most of the markers revealing five alleles or more. The number of alleles at each locus varied from 3 (AC07 and AC08) to 8 (AC23) with a mean of 5.62 alleles per locus. The polymorphism information content (PIC) value, which is a measure of polymorphism for a marker locus, varied from 0.46 (AC08) to 0.84 (AC01) among the SSR markers loci, the average being 0.70. The effective number of alleles per locus (Ne) ranged from 1.28 to 3.11 with a mean of 2.31. The marker AC07 had the lowest effective number of alleles per locus and the marker AC29 had the highest numbers of effective alleles. Shannon's Information Index ranged from 0.43 (AC07) to 1.33 (AC23) with a mean of 0.97. The observed heterozygosity (H_o) values ranged from 0.25 to 1.00 with an average of 0.73. The expected heterozygosity (H_e) values ranged from 0.22 to 0.69 with an average of 0.54. The degree of genetic differentiation among the subpopulations (F_{ST}) ranged from 0.11 (AC06) to 0.66 (AC01) with a mean of 0.32. The gene flow (N_m) values ~~were~~ ranged from 0.12 (AC01) to 1.96 (AC06) with a mean gene flow value of0.52. The lowest gene flow value was observed in the marker AC01 and the highest gene flow was exhibited by ~~the~~ AC06. The dendrogram constructed using similarity coefficient values by following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) formed two major clusters with cluster I consisting of all the 16 cultivated varieties and 2 wild species*viz.*, *Areca triandra* and *Areca concinna* and the

second major cluster comprised of related genera viz., *Normanbyanormanbyii* and *Actinorhytiscalapparia*. ~~Highest~~ The highest genetic similarity was observed between the cultivated varieties, Kahikuchi and Mohitnagar and Nalbari and Sumangala, and the lowest similarity value of 0.33 was found between the related genera, *Normanbyanormanbyii* and the cultivated variety, Sreemangala.

Key words: Arecanut, genetic diversity, SSR markers, cultivated varieties, wild species, related genera

1. INTRODUCTION

Plantation crops in India are one of the major segments of the horticulture sector and the mainstay of agrarian economies in many states and Union Territories of the country [1]. In our country, arecanut is one of the important plantation crops which plays an important role in the religious, social, cultural and economic life of people. Arecanut (*Areca catechu* L.), also known as Pinang or Betel nut, is an ancient crop domesticated in Southeast Asia and it is widely used in various Asian and Pacific cultures [2]. In the world, arecanut is grown in an area of about 12.26 lakh hectares with production and productivity of 17.96 lakh tonnes and 2518.63 kg/ha, respectively [3]. India ranks first both in area and production of arecanut. In our country, arecanut is grown in an area of about 5.27 lakh hectares with production of 9.04 lakh tonnes and productivity of 1715.7 kg/ha, respectively [3].

Arecanut is believed to have numerous medicinal values [4] [5]. It has an important place in the ancient Indian system of medicine such as Ayurveda, Unani and Homeopathy [6] [7] and in clinical practices in certain other countries such as Philippines, China and other South and South-East Asian countries [8] [9] [10]. It was also reported that all the alkaloids present in arecanut possess drug-like properties [11]. Most of the folklore medicinal properties of arecanut are now validated and authenticated with proper scientific data [12]. Arecanut plant parts are used for the preparation of household and other articles such as cups, plates, ply boards, hard boards, hats etc. Arecanut extracts used for the preparation of areca tea, soap, wine etc. Arecanut leaf sheath is also used as alternate fodder for livestock [13] [14].

Identification of cultivars and estimation of genetic diversity using phenotypic markers have several limitations, especially in perennial crops [15]. Molecular differences, which can be detected using DNA and protein-based markers, are more authentic and unaffected by environmental factors [15]. However, the choice of markers to be used depends on the availability of genetic information about the genome sequence, cost for marker

development, ease of documentation and polymorphism [16]. Among the DNA based markers, microsatellite markers are found to be the more suitable, because they are co-dominant, exhibit high polymorphism, reproducible, can be detected easily, abundant in number etc. The application of the microsatellite loci may provide a powerful tool for assessing ~~the~~ human group interaction and the co-migration of crop carry-on when *A. catechu* is brought as a significant hobby gift, playing important roles in ritual and subsistence life across Southeast Asia [2].

Even though arecanut is an important plantation crop giving high economic returns, very less studies have been done on analysis of genetic variation using simple sequence repeat markers. Hu et al. [2] characterized the polymorphic microsatellite loci from arecanut germplasm which resulted in the isolation of nine novel microsatellite loci from *A. catechu* germplasm. Nagaraja et al. [17] studied genetic diversity among 24 arecanut germplasm by employing SSR markers. Kiran Kumar et al. [18] studied genetic diversity among 25 arecanut germplasm by employing microsatellite markers. The large scale study of estimation of genetic diversity and population structure in arecanut (*Areca catechu* L.) was done by Bharathet al. [19]. They studied genetic relationships existing amongst 60 arecanut accessions by employing nine microsatellite primers. A total of 42 alleles were detected, with an average of 4.66 alleles per locus. The PIC values ranged from 0.50 to 0.78, with an average of 0.69. Expected heterozygosity (*He*) was highest for the exotic accessions (0.31), moderate for Konkan- I and II accessions, while the least heterozygosity was observed for the accessions from Maidhan tract (Karnataka) and Andaman and Nicobar Islands. ~~Mean~~ The mean fixation index (F_{ST}) of 0.28 indicated a high level of population differentiation.

ICAR-Central Plantation Crops Research Institute has the largest collections of arecanut germplasm in the world. Understanding the genetic diversity present among the crop species is very important for breeding programmes. Therefore, this study was carried out to elucidate the genetic variation among arecanut varieties, wild species and related genera maintained in the field gene bank by employing microsatellite markers.

2. MATERIALS AND METHODS

2.1 Plant materials

Twenty arecanut genotypes comprising of 16 cultivated types (including 13 cultivated varieties, two dwarf hybrids and a natural mutant), two wild species (*Areca triandra*and *Areca concinna)* and wild genera *(Normanbyanormanbyii*and*Actinorhytiscalapparia),* were used for the present study. These accessions are conserved and maintained in the National

Arecanut Gene Bank at ICAR-Central Plantation Crops Research Institute, Regional Station, Vittal, Karnataka State, India (Latitude: 12º 15^1 N, Longitude: 75º 25^1 E), which is considered to be the largest arecanut germplasm collection in the world. The detailed information about the material used in this study is given in Table 1.

2.2 Extraction of genomic DNA

Young spindle leaf tissues collected from cultivated varieties, wild species and two other ~~two~~ generaofarecanut were used for DNA extraction. Total genomic DNA of the arecanut samples was isolated by using DNeasy plant mini kit (Qiagen) as per the protocol provided in the kit. To check purity and intactness of DNA, the genomic DNA was run in 0.8% agarose gel stained with ethidium bromide following the protocol of Sambrooket al. [20] and was visualized in a gel documentation system (Gel Doc XR, Bio-Rad, CA, USA). Quantification of DNA was done by using a spectrophotometer.

2.3 Molecular markers analysis

Simple Sequence Repeat primer pairs developed by Hu et al. [2] were used for molecular marker analysis. The sequences of the primers used are given in Table 2. PCR reactions were conducted in volumes of 20 µl containing 35 ng genomic DNA, 0.2 µM each of forward and reverse primers (Bangalore Genei, India), 50 µM of each dNTPs (Bangalore Genei, India), 1X buffer [10 mMTris-HCl (pH 8.3), 50 mMKCl, 1.5 mM $MgCl_2$] and 0.3 Unit of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in an thermal cycler (Eppendorf Mastercycler) with an initial denaturation at 94ºC for 5 min followed by 40 cycles of denaturation for 1 min at 94ºC, 2 min at the different annealing temperatures standardized for the individual SSR locus (Table 2) and extension for 2 min at 72ºC with a final extension for 5 min at 72ºC. After amplification, a volume of 3 µl of loading dye was added to each of the amplified product.

The amplification products were subjected to electrophoresis and resolved in a 3% high resolution agarose gel using 1X TBE buffer and the gel was run at 80V for 4-5 h in an electrophoresis unit. The ethidium bromide stained gels were visualized and photographed using a gel documentation and analysis system (Gel Doc XR, Bio-Rad, CA, USA). Amplification reactions were repeated thrice for checking reproducibility.

2.4 Data analysis

Each band generated by SSR primers was considered as an independent locus. Clearly resolved, unambiguous bands were scored visually for their presence or absence. The alleles

scored were compared with a 100 bp molecular ladder (Bangalore Genei, India). The scores are obtained in the form of a matrix with '1' and '0', which indicate the presence and absence of bands respectively in each sample. The binary data scored was used to construct a dendrogram. The genetic associations between genotypes were evaluated by calculating the Dice similarity coefficient for pairwise comparisons based on the proportions of shared bands produced by the primers [21]. The similarity matrix was generated using the NTSYS-PC v.2.0 software [22]. The similarity coefficients were used for cluster analysis, and dendrogram was constructed by Unweighted Pair-Group Method with Arithmetic Average (UPGMA) [23].

The average Polymorphism Information Content (PIC) ~~were~~ was calculated by applying the formula given by [24] and [25].

PIC = $1 - \Sigma f i^2$, Where i = 1 – n, f_i is the frequency of the i^{th} allele andn represents the number of alleles. The number of alleles refers to the number of scored bands. The frequency of an allele was obtained by dividing the number of accessions where it was found by the total number of accessions. The PIC value provides an estimate of the discriminating power of a marker.

Observed number of alleles, effective number of alleles (N_e), Shannon's Information Index (I) and F-Statistics were worked out for eight microsatellite loci using the software Population Genetic Analysis (POPGENE) version 1.31 [26]. The expected and observed heterozygosity across the 20 arecanut accessions were worked out using the software GDA (Genetic Data Analysis) [27].

3. Results and discussion

3.1 Polymorphism of Simple Sequence Repeat markers

Genetic diversity parameters, such as number of alleles per locus (N_a), effective number of alleles (N_e), Shannon's information index (I) and polymorphic information content (PIC) are presented in Table 3. A total of 45 alleles were detected with most of the markers revealing five alleles or more. The number of alleles at each locus varied from 3 to 8 with a mean of 5.62 alleles per locus. Similar results were obtained by Bharath et al. [19], they detected a total of 42 alleles with an average of 4.67 alleles per locus. Study by Hu et al. [2] observed 5 to 15 alleles per locus with average of 11.44 alleles per locus. The lowest numbers of alleles per locus were detected from the markers AC07 and AC08. The highest number of alleles per locus was detected from the marker AC23. The effective number of alleles per

locus (N_e) ranged from 1.28 to 3.11 with a mean of 2.31. The marker AC07 had the lowest effective number of alleles per locus and the marker AC29 had the highest numbers of effective alleles. Shannon's Information Index ranged from 0.43 (AC07) to 1.33 (AC23) with a mean of 0.97. The polymorphism information content (PIC) value, which is a measure of polymorphism for a marker locus, varied from 0.46 (AC08) to 0.84 (AC01) among the SSR markers loci, the average being 0.70. Moderate to higher level of polymorphic information content was observed. SSR primers showed an average PIC value of >0.5, which indicates that the primers are highly informative. PIC values ranged from 0.50 to 0.78, with an average of 0.69 was recorded by Bharath et al. [19]. SSR banding profiles generated using primer AC01 for the arecanut varieties, wild species and related genera are shown in the Figure 1.

The observed heterozygosity (H_o) values ranged from 0.25 to 1.00 with an average of 0.73 and the expected heterozygosity (H_e) values ranged from 0.22 to 0.69 the average being 0.54 (Table 4). Hu et al. [2] recorded the expected and observed heterozygosities ranged from 0.71 to 0.94 and from 0 to 0.88, respectively. Bharath et al. [19] reported that the expected heterozygosity *(H_e)* was highest for the exotic accessions (0.31), moderate for Konkan- I and II accessions, while the least heterozygosity was observed for the accessions from Maidhan tract (Karnataka) and Andaman and Nicobar Islands. The degree of genetic differentiation among the subpopulations (F_{ST}) ranged from 0.11 to 0.66 with a mean of 0.32, indicating a high level of population differentiation (Table 4). The lowest and highest degree of genetic differentiation among the subpopulations was recorded from the SSR markers AC06 and AC01, respectively. ~~Mean~~ The mean fixation index *(F_{ST})* of 0.28 indicated a high level of population differentiation in Bharath et al. [19] study. Negative inbreeding coefficient (F_{IS}) was noticed for all the microsatellite markers. For most of the loci negative degree of genetic differentiation (F_{IT}) was observed except AC01 microsatellite marker. The gene flow (N_m) values ~~were~~ ranged from 0.12 to 1.96 with a mean gene flow value of0.52. The lowest gene flow value was observed in the marker AC01 and the highest gene flow was exhibited by the AC06 microsatellite (Table 4).

3.2 Cluster analysis

The dendrogram was constructed using similarity coefficient values by following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram formed two major clusters, with cluster I consisting of all the 16 cultivated varieties and 2 wild species, *viz.*, *Areca triandra* and *Areca concinna* and the second major cluster comprised of related genera namely, *Normanbyanormanbyii* and *Actinorhytiscalapparia*(Figure 2).The

percentage of similarity coefficient varied from 0.47 to 1.00 between the cultivated varieties, wild species and related genera indicating the existence of a moderately high level of genetic diversity among the accessions studied. ~~Highest~~ The highest genetic similarity was observed between the cultivated varieties, Kahikuchi and Mohitnagar and Nalbari and Sumangala and the lowest similarity value of 0.33 was found between the related genera, *Normanbyanormanbyii* and the cultivated variety, Sreemangala. Kahikuchi and Mohitnagar varieties are selections from the indigenous arecanut accessions collected from Assam and West Bengal, respectively. Nalbari and Sumangala varieties are developed from the accessions obtained from Assam (India) and Indonesia, respectively. TheSreemangala variety is developed from the accession obtained from Singapore. In Bharathet al. [19] investigation, the UPGMA cluster analysis grouped the accessions into two major clusters- the indigenous Konkan accessions clustered separately in a distinct cluster along with Maidhan accessions. In the second major cluster, indigenous accessions from North East India formed a unique sub-cluster, while accessions from Andaman and Nicobar Islands were grouped with exotic accessions.

The value of genetic diversity, in its various forms, has been extensively discussed in literature [28] [29]. Genetic diversity is a natural buffer mechanism against the genetic vulnerability which has been built into the genetic structure of traditional cultivars [30]. Landraces may contain co-adapted gene complexes that have evolved over decades [31] and are the most important of the plant genetic resources. The great wealth of genetic diversity still existing in plant gene pools holds vast potential for current and future uses of humankind [31]. Advanced cultivars, genetic stocks and wild relatives of crop plants play an important role in crop improvement and therefore need to be preserved [32]. In general terms, agricultural biodiversity provides many goods and services of environmental, economic, social and cultural importance, these environmental goods and services also contribute to sustainable livelihoods in a number of ways [33].

4. Conclusion

As wild species/relatives of crop plants are reservoirs of genetic variation and play an important role in crop improvement. Investigations ~~on~~ into thegenetic diversity of any crop are imperative in germplasm characterization, conservation and use in breeding programmes. Therefore, the results of the present investigation can be utilized for germplasm characterization and utilization of invaluable germplasm for formulating arecanut improvement programmes.

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Table 1.Arecanut varieties, wild species and related genera used in the study

Sl. No.	Variety/ wild species/ related genera	Status

1	Mangala	Varieties developed and released by evaluating exotic germplasm
2	Sumangala	
3	Sreemangala	
4	Swarnamangala	
5	Mohitnagar	Varieties/hybrids developed and released by evaluating indigenous germplasm
6	Kahikuchi	
7	Nalbari	
8	Kodinar	
9	VTLAH1 (Dwarf hybrid)	
10	VTLAH2 (Dwarf hybrid)	
11	Hirehalli Dwarf	Natural dwarf mutant
12	S.K. Local	Local indigenous varieties
13	SAS1	
14	Sagar	
15	Shrivardhana	
16	Thirthahalli	
17	*Areca triandra*	Wild species of arecanut
18	*Areca concinna*	
19	*Normanbyanormanbyii*	Related genera of arecanut
20	*Actinorhytiscalapparia*	

Table 2. List of arecanut-specific microsatellite primer pairs with their sequences, repeat motif and annealing temperature

Sl. No	Primer name	Sequence (5'-3')		Repeat motif	Tm (°C)
		Forward primer	Reverse primer		
1	AC01	GCGTGATCCACATGTACCTT	TTCGGGGAATACTGAGATGG	$(AG)_{12}$	50
2	AC06	AGCCTGGAAGGGTTCTCTTT	TGGGAAACGAGTGAAGAAGG	$(TC)_7G(GT)_4$	50
3	AC07	CCCATATGTTTGGGAGCAAC	AAACATGACACATGGGGTGA	$(TC)_{10}(TG)_3$	50

4	AC08	TGAAGACAGAAGACCCGCA	TGCATCCATGGAGTTGTGTT	$(TC)_8(TG)_5$	50
5	AC14	ATGTATCTGCTCCCCCTGTG	CGGGCTGGTCTAGATAAGGA	$(AC)_9(AG)_7$	50
6	AC23	TCACCCATCCTTTTTGAATA	CCGGTACCAAGACGGTGAT	$(CT)_{17}$	50
7	AC29	GCAATGCAGTCCTTTTGTAT	CTCGTAGTTTGGGTGGATTA	$(CT)_{14}(GT)_{20}$	55
8	AC30	ATTGGCCGATCAGCAAGTAA	TGCAATGCAGTCCTTTTGTA	$(AC)_{16}(AG)_{38}$	55

Table 3. Diversity statistics for the SSR markers

Sl. No.	Locus	Allele size (bp)	No. of alleles (N_a)	Effective no. of alleles (N_e)	Shannon's information index (I)	Polymorphism information content (PIC)
1	AC01	251–562	5	1.7008	0.8111	0.84
2	AC06	100–347	7	2.2923	0.9045	0.65
3	AC07	389–708	3	1.2882	0.4393	0.65
4	AC08	355–501	3	2.1978	0.8557	0.46
5	AC14	316–794	6	2.0460	0.9608	0.82
6	AC23	135–955	8	3.0769	1.3336	0.65
7	AC29	158–355	6	3.1128	1.2871	0.78
8	AC30	105–309	7	2.8369	1.2371	0.76
	Mean			**2.3190**	**0.9786**	**0.70**

Table 4. Observed heterozygosity (H_o), expected heterozygosity (H_e) and gene flow for the microsatellite primer pairs

Sl. No.	Locus	H_o	H_e	F_{IS}	F_{IT}	F_{ST}	N_m
1.	AC01	0.3889	0.4238	-1.0000	0.3317	0.6659	0.1254
2.	AC06	1.0000	0.5782	-1.0000	-0.7738	0.1131	1.9608
3.	AC07	0.2500	0.2295	-1.0000	-0.1173	0.4413	0.3165
4.	AC08	0.9000	0.5590	-1.0000	-0.6514	0.1743	1.1842

5.	AC14	0.6000	0.5244	-1.0000	-0.1736	0.4132	0.3550
6.	AC23	1.0000	0.6923	-1.0000	-0.4815	0.2593	0.7143
7.	AC29	0.8500	0.6962	-1.0000	-0.2523	0.3738	0.4187
8.	AC30	0.9000	0.6641	-1.0000	-0.3900	0.3050	0.5696
	Mean	**0.7361**	**0.5459**	**-1.0000**	**-0.3567**	**0.3217**	**0.5272**

F_{IS}: Coefficient of Inbreeding among individuals in the subpopulations; F_{IT}: Degree of Genetic Differentiation among the total populations; F_{ST}: Degree of Genetic Differentiation among the subpopulations; N_m:Gene Flow.

Figure 1. SSR banding profiles generated using primer AC01 for the arecanut varieties, wild species and related genera standard 100 bp ladder (M) used as a reference.

Figure 2.A UPGMA dendrogram based on SSR data for the cultivated varieties, wild species and related genera of arecanut.