Estimation of out-crossing rates in populations of West Coast Tall cultivar of coconut using microsatellite markers

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Abstract

Understanding of mating system of a plant species has fundamental importance for formulation of genetic conservation strategies and breeding programmes. The pattern of gene flow, *via* pollen, has a profound influence on the genetic structure within a population. Various genetic parameters, obtained from molecular marker studies, can be used to assess estimates of mating system. The aim of this study was to estimate the rate of outcrossing in West Coast Tall (WCT) cultivar of coconut, which is predominant in India, using microsatellite simple sequence repeats (SSR). Two WCT mother palms and their 88 progenies, collected as embryos for five months, were screened using 15 highly polymorphic microsatellite primers. The mating parameters were estimated using mixed mating model (MLTR software) and the extents of similarity between the mother palms and their progenies were analyzed using the NTSYS software. The percentage similarity between the mother palm and its progenies, as deduced using microsatellite data, ranged from 55 to 74 per cent. The progenies were also analyzed using a RAPD primer capable of distinguishing Tall and Dwarf palms. All the progenies were found to possess the Tall-type marker indicating that the pollen was derived from Tall palms in all the cases. The results revealed the WCT cultivar to be pre-dominantly out-crossing and indicated that proper sampling and indicated that proper sampling and breeding strategies are required to sustain the high genetic diversity found.

Keywords: Coconut, microsatellite, out-crossing, WCT

Introduction

Coconut (*Cocos nucifera* L.) is the most widely and naturally distributed palm tree, which is extensively cultivated around the world and is considered to be one of the most important tropical species used by man (Persley, 1992). Earlier researchers believed southeast Asia to be the center of origin of the species due to the great morphological variability, the large number of popular/local names, plant uses, and the number of associated insects in that region (Harries, 1978). It has been suggested that the spreading of the species throughout diverse regions of the world occurred naturally by the buoyant fruits, carried by oceanic currents across southeast Asia to the Pacific and Indian Oceans, and also by human migration during the colonization of Asia and America. It is now considered that coconut palm might have originated independently in the Pacific and also Indo-Atlantic oceanic basins (Gunn *et al*., 2011).

Understanding the mating system of the species is fundamental in formulating programmes for genetic improvement and germplasm conservation, since it permits the outlining of strategies that optimize the sampling of genetic variability and the adoption of biometric-statistical models appropriate for the estimation of genetic parameters. Therefore, it is important to establish strategies for the effective conservation of any species. The mating system, together with the mechanism of pollen and seed dispersal, determines the genetic structure of populations (Freitas *et al*., 2004).

Two main groups of coconut palm trees, the Tall (*Typica*) and the Dwarf (*Nana*), are known.

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Since Tall cultivars are protandrous, they are believed to be largely cross-pollinated. Dwarf cultivars, are largely self-pollinating as opposed to the Talls. Classical breeding of coconut palm is mainly based on selection of phenotypic traits and artificial hybridization, involving varietal crosses between Tall and Dwarf forms. Research efforts to enhance coconut production and productivity commenced in the early part of 20th century and in spite of its perennial habit, breeding system, long pre-bearing age, heterozygous nature, low rate of multiplication and requirement of large area for experimentation, substantial progress has been made by concerted breeding efforts (Nair *et al.*, 1988).

Most of the breeding initiatives in coconut have relied on mass selection, mostly using open pollination, as a means of coconut improvement. The selection criteria mainly included yield of copra per tree, number of fruits produced and net copra content. However, the results derived from various studies on coconut improvement (mostly for yield) using mass selection were highly contradictory, the differences being attributed to the reproduction characteristics of the Tall coconut genotypes, which although are preferentially allogamous (Bourdeix, 1999). However, natural selfing has also been reported in Tall genotypes with the rate of selfing increasing with the rhythm of inflorescence production, which, in turn, is dependent on the individual vigour of the tree and also on climatic factors (Bourdeix, 1999; Ratnambal *et al.*, 2003). During selection of coconut palms performing well, their progeny may be subjected to inbreeding depression if trees with a higher tendency towards selfing are selected (Bourdeix, 1999). The rhythm of inflorescence emission has also been known to show seasonal variations.

Satyabalan and Lakshmanachar (1960) made a comparison of selfed (S_1) and open-pollinated (OP) progenies of 18 Tall coconut ecotypes and reported reduction of vigour, number of fruits produced and copra content per nut due to selfing. They also reported differences in the intensity of the inbreeding depression between the 18 ecotypes. A study of cytological behaviour of the S_1 progenies of these ecotypes revealed an increase in pollen sterility and chromosomal aberrations (Nambiar *et al.*, 1970), which was accompanied by a reduction

in chiasma frequency and corresponding decrease in recombination frequency. The spread of coconut, in conjunction with founder effects, might have resulted in formation of naturally inbred populations and induced genetic drift. As a result, populations capable of resisting out-breeding, *i.e.*, those subjected to natural inbreeding; possess limited variability and consequently, only lesser chances of within-population improvement (Bourdeix, 1999). Studies on S_1 and OP progenies of 17 non-selected Sri Lankan Tall palms revealed inbreeding depression for leaf number and flowering precocity (Liyanage, 1969).

A thorough study of coconut pollination biology will be of significance in designing of efficient germplasm selection and conservation strategies which would help in laying out seed gardens for production of hybrid seeds (Regi Thomas and Josephrajkumar, 2013). Pollination of coconut has been reported to be effected mainly by insects (Child, 1974) and also by wind. A study by Manthriratna (1971) in Sri Lanka revealed that coconut pollen could be found at a distance of 180 m from the emission source.

There is a chance that self-pollination may also occur in Tall coconut cultivars, even though they are classified as predominantly allogamous. A study on Tall coconut palms by Patel (1938) revealed the existence of seasonal variations in the palm reproductive behaviour. At least 75% of the trees showed overlapping between male and females phases during April which may allow for selfing, whereas, no overlapping took place during November. With the commencement of dry season in Cote D'Ivoire, quick production of inflorescences was observed, which increases the chances of self-pollination (Bourdeix, 1999). Also, significant differences were observed in the rate of self pollination among the individual Tall palms: palms which were high yielding produced more inflorescences and, consequently higher selfing rates (Bourdeix, 1999).

Till date, there has not been any systematic study on mass selection using open pollination in coconut, even though it is being utilized in coconut improvement programmes. Mass selection using open pollination will definitely lead to high variability in results because of the absence of control of the pollen origin. In spite of these

divergent results, mass selection is adopted widely because of its simplicity of application.

In recent years, there has been an enormous surge of interest in molecular marker technologies and their application to study the structure and evolution of natural populations. One of the most important aspects of population structure is described by the behavior of a mating system that has important consequences for a population's evolution and the efficacy of natural selection (Charlesworth, 2003). For example, selfing and other forms of inbreeding produce higher homozygote frequencies in the population than expected under random mating, thus leading to the reduction of the population's effective size (Gao *et al*., 2007). By genotyping multiple markers in samples of individuals collected from the population, it is possible to estimate the rates of outcrossing and inbreeding from the segregation patterns of the markers (Barriere and Felix, 2005). The precise characterization of the outcrossing rate can benefit from the simultaneous analysis of multiple markers while integrating gene co-segregation and non-random association into the analysis.

Of the varieties of coconut available in India, the West Coast Tall (WCT) cultivar is the most common. This variety of coconut is extensively cultivated in all the important coconut tracts of India (about 95 per cent of the area) and is of commercial importance. WCT is found to grow well in littoral sand as well as in the interior lateritic soils and up to an altitude of about 3,000 feet (915 meters) above sea level. It has been cultivated in India from very ancient times and may, therefore, be considered as indigenous to the country. WCT is a long living, hardy, multi-purpose palm, yielding nuts, copra, oil, and coir fibre of good quality. The tree also yields, on tapping good quantity and quality of 'neera' which can be fermented into toddy or converted into jaggery or palm sugar. The objective of this work was to estimate outcrossing rates in two palms of WCT cultivar and their OP progenies using highly polymorphic microsatellite markers.

Materials and methods

Plant materials

Two palms of WCT cultivar of coconut planted at Central Plantation Crops Research Institute CPCRI, Kasaragod, India were chosen for the study.

Bunches were tagged and harvested from the palms at intervals of 45 days. Zygotic embryos were scooped out along with a portion of the endosperm using a cork borer from mature, dehusked and split coconuts (11-12 months old). The embryos were extracted from the endosperm plug using a scalpel. Under aseptic conditions, the embryos were washed in 50 per cent chlorine water for 20 minutes and then rinsed four times with sterile distilled water. The sterilized embryos were inoculated into plain Y3 medium containing 3 per cent sucrose, $1g\ L^{-1}$ activated charcoal and 0.55 per cent (w/v) agar (Eeuwens, 1976). The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl before autoclaving for 20 minutes at 121°C. The cultures were incubated in the dark for a month at 27±2°C. The number of embryos collected from the two palms is given in Table 1.

Table 1. List of samples used in the present study

Palm No.	Month	No. of nuts per embryos harvested
WCT 280	December 2011	11
	January 2012	10
	February 2012	11
	March 2012	9
WCT 238	December 2011	12
	January 2012	11
	February 2012	12
	March 2012	12
	Total embryos collected	88

DNA extraction

After a month in culture, when the embryos increased in size, they were ground in liquid nitrogen and DNA was extracted from the embryos using the DNeasy mini kit (QIAGEN). DNA was also extracted from spindle leaves of the two mother palms using a rapid SDS-procedure (Rajesh *et al*., 2013).

SSR analysis

A set of 15 hyperpolymorphic coconut SSR markers (Table 1), distributed in different coconut chromosomes were used for the analysis. PCR reactions were conducted in volumes of 20 µL containing 35 ng genomic DNA, 0.2 µM each of

forward and reverse primers, 50 µM of each dNTPs (M/s Bangalore Genei Pvt. Ltd., Bangalore), 1X buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) and 0.3 Unit of *Taq* DNA polymerase (M/s. Bangalore Genei Pvt. Ltd., Bangalore). PCR amplifications were performed on a BIORAD gradient thermal cycler with a PCR profile of 94 ℃ for 5 min followed by 30 cycles of 1 min at 94 ℃, 2 min at the different annealing temperatures standardized for the individual SSR locus, and 2 min at 72 ℃ with a final extension for 5 min at 72 ℃. After amplification, a volume of 3 µL of loading buffer (98% formamide, 10 mM EDTA, 0.005% each of xylene cyanol and bromophenol blue as tracking dyes) was added to each of the amplified product. The amplified products were run on 3 per cent high resolution agarose gel, stained with ethidium bromide and were visualized in a gel documentation system.

RAPD analysis

RAPD analysis was performed using a polymorphic primer OPBA03 obtained from Operon Technologies Inc. (Alameda, CA, USA) on the two mother palms and their progenies. This primer is capable of distinguishing Tall, Dwarf and Hybrid palms (Rajesh *et al.*, 2014). PCR reactions were conducted in volumes of 15 µL containing 35 ng genomic DNA, 10 µM primer, 10 mM of each dNTPs (M/s MBI Fermentas), 10X buffer (10 mM Tris-HCl (pH 8.3) and 5 Units of *Taq* DNA polymerase (M/s MBI Fermentas). The amplification conditions were: an initial denaturation step (94 °C for 5 min), followed by 39 cycles at 94 °C for 1 min, 42 °C for 1 min and 72 °C for 1 min 30 sec, terminating with a final extension at 72 °C for 10 min. The amplified products were run on 1.2 per cent agarose gel, stained with ethidium bromide and photographed on a digital gel documentation and image analysis system. The bands were scored visually.

Data analysis

The alleles were scored individually based on comparison with the molecular ladder. The size of the amplicons was compared using a 100 bp ladder (M/s Bangalore Genei, India). Each band generated by SSR primers was considered as an independent locus. Clearly resolved, unambiguous bands were

Table 2. Coconut-specific SSR primers used in the current study

Sl. No.	Primer name	Sequence (5'-3')	Tm (°C)
1	CnCir87F	ATAACATCCTCCAACCTG	55
	CnCir87R	GACTGAATCCAACCCTT	
2	CnCir74F	GAGATCCTCACCTCCAC	52
	CnCir74R	CGGCAACAAAGAGAAC	
3	CnCirG11F	AATATCTCCAAAAATCATCGAAAG	52
	CnCirG11R	TCATCCCCACACCCTCCTCT	
4	CnCirE4F	GCATGGTATTCGGATTTG	54
	CnCirE4R	ATGGTTCAGATTTGGACAGT	
5	CnCirE10F	TTGGGTTCCATTTCTTCTCTCATC	59
	CnCir E10R	GCTCTTTAGGGTTCGCTTTCTTAG	
6	CnCirC3F	AATATCTCCAAAATCATCGAAAG	59
	CnCirC3R	GTGGGGCATGAAAAGTAAC	
7	CnCir 2F	AGTCCTAAAAGTGTTGGC	56
	CnCir 2R	GTAATCCTATGGCTGCTT	
8	CnCir B12F	GCTCTTCAGTCTTTCTCAA	57
	CnCir B12R	CTGTATGCCAATTTTTCTA	
9	CnCir E2F	TCGCTGATGAATGCTTGG	55
	CnCirE2R	GGGGCTGAGGGATAAACC	
10	CnCirB6F	GAGTGTGTGAGCCAGCAT	59
	CnCirB6R	ATTGTTCACAGTCCTTCCA	
11	CnCir56F	AACCAGAACTTAAATGTCG	51
	CnCir56R	TTTGAACTCTTCTATTGG	
12	CNZ10F	CCTATTGCACCTAAGCAATTA	54
	CNZ10R	AATGATTTTCGAAGAGAGGTC	
13	CNZ05F	CTTATCCAAATCGTCACAGAG	50
	CNZ05R	AGGAGAAGCCAGGAAAGATTT	
14	CNZ04F	TATATGGGATGCTTTAGTGGA	52
	CNZ04 R	CAAATCGACAGACATCCTAAA	
15	CNZ17F	ATGTAAAGAAAGTAGGGAGGC	60
	CNZ17 R	CATAGGTTATCATGCAGAGCT	

scored visually for their presence or absence with each primer. The scores were obtained in the form of a matrix with '1' and '0', which indicate the presence and absence of bands respectively in each sample.

Genetic similarity between the mother palms and their progenies was estimated for each month using similarity matrix, generated by calculating Dice's similarity coefficient (Dice, 1945). These similarity coefficients were then used for cluster analysis and dendogram was constructed by the unweighted pair-group method (UPGMA) (Sneath

and Sokal, 1973) using the software package NTSYS-pc version 2.02 (Rohlf, 1998).

For the microsatellite data, the number of alleles, number of effective alleles, Shannon's information index, the observed and expected heterozygosity, Wright's fixation indices, gene flow were estimated using the software POPGENE version1.32 (Yeh *et al*., 1999). We tested data sets for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) in GENEPOP v4.0 (Rousset, 2008), using a Markov chain approximation to exact tests and likelihood-ratio tests, respectively. Deviations from HWE were estimated using both the exact test and the FIS statistic estimations, using Markov chain Monte Carlo (MCMC) runs for 1000 batches, each of 2000 iterations, with the first 500 iterations discarded before sampling. Whenever multiple testing was performed, probability values were corrected using standard Bonferroni corrections (Rice, 1989).

In order to estimate the variance between the groups of populations, pooled sample structuring was estimated using analysis of molecular variance (AMOVA) and 20,000 permutations implemented in Arlequin v 3.5.1.2 (Excoffier *et al*., 1992).

The mating system was analyzed based on the model of crossing mix of Ritland and Jain (1981), with the aid of Multilocus MLTR software version 3.4 available at http://www.genetics.forestry.ubc.ca/ritland/programs.html (Ritland, 2002). The genetic parameters estimated were: (a) Multilocus outcrossing rate of the population (tm) by the method of Expectation-Maximization (EM); (b) The average population single locus (unilocus) outcrossing rate (ts); (c) Correlated paternity (rp) and; (d) The multilocus inbreeding coefficient or Wright's fixation indices of maternal generation (Fm). The variances of the estimates were obtained by bootstrapping (1000 bootstraps). The estimation method was based on the maximum likelihood equations (Ritland, 1983). This estimation process was used according to the following conditions: the genotypes of the female parents are known and the gene frequencies in the pollen pool are unknown.
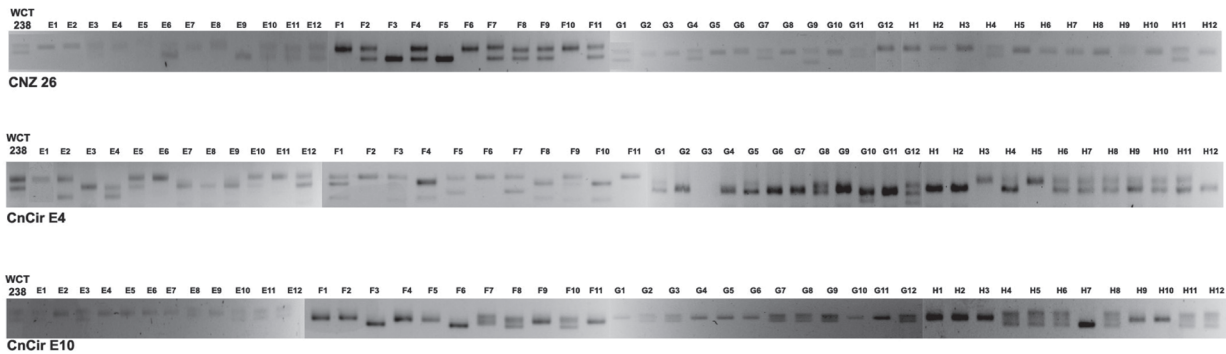

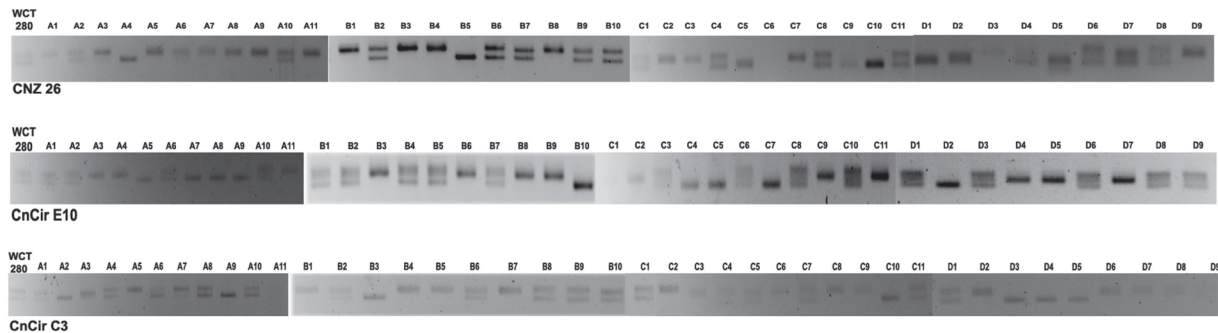**Fig. 1a. WCT 238 and its OP progenies**

Fig. 1b. WCT 280 and its OP progenies

Table 3. Observed number of alleles (N$_a$), effective number of alleles (N$_e$) and Shannon's information index (I) for the 15 microsatellite loci

| Sl No.	Locus	N$_a$	N$_e$	I
1.	Cncir87	3.0000	1.9039	0.8173
2.	CnCirG11	3.0000	1.6569	0.6502
3.	CnCirE4	3.0000	1.7916	0.7600
4.	CnCir136	3.0000	2.2310	0.8895
5.	CnCirE10	3.0000	2.7607	1.0564
6.	CnCirB12	2.0000	1.3006	0.3927
7.	CnCir3	3.0000	2.5983	1.0254
8.	CnCir56	2.0000	1.9961	0.6922
9.	CnCir74	3.0000	1.9948	0.8547
10.	CnCir2	2.0000	1.1421	0.2449
11.	CNZ26	3.0000	2.5154	1.0081
12.	CNZO4	3.0000	1.7108	0.7377
13.	CNZO5	2.0000	1.3465	0.4256
14.	CNZ10	3.0000	1.1443	0.2824
15.	CNZ17	3.0000	2.0321	0.7434
	Mean	2.7333	1.8750	0.7054
	St.Dev	0.4577	0.5114	0.2621

N$_a$: Observed number of alleles; N$_e$: Effective number of alleles (Kimura and Crow, 1964); I: Shannon's information index (Lewontin, 1972)

Results and discussion

Allele richness of SSR loci

Fifteen polymorphic SSR markers were used to amplify DNA of two WCT palms and their 88 progenies (Fig.1a and 1b). A total of 41 alleles were detected with all the markers revealing two alleles or more with a mean of 2.7 alleles per locus. The effective number of alleles per locus (N$_e$) ranged from 1.3 (CnCirB12) to 2.7 (CnCirE10) with a mean of 1.875. Shannon's information index ranged from 0.24 (CnCir2) to 1.05 (CnCirE10) with a mean of 0.705 (Table 3).

Genetic similarity between the mother palms and their progenies were estimated for each month using similarity matrix, generated by calculating Dice's similarity coefficient and the similarity coefficients were then used for cluster analysis and dendrogram was constructed by UPGMA . The percentage similarity between the mother palm and their progenies, as deduced using microsatellite data, ranged from 55 to 74 per cent (Fig. 2 a,b).
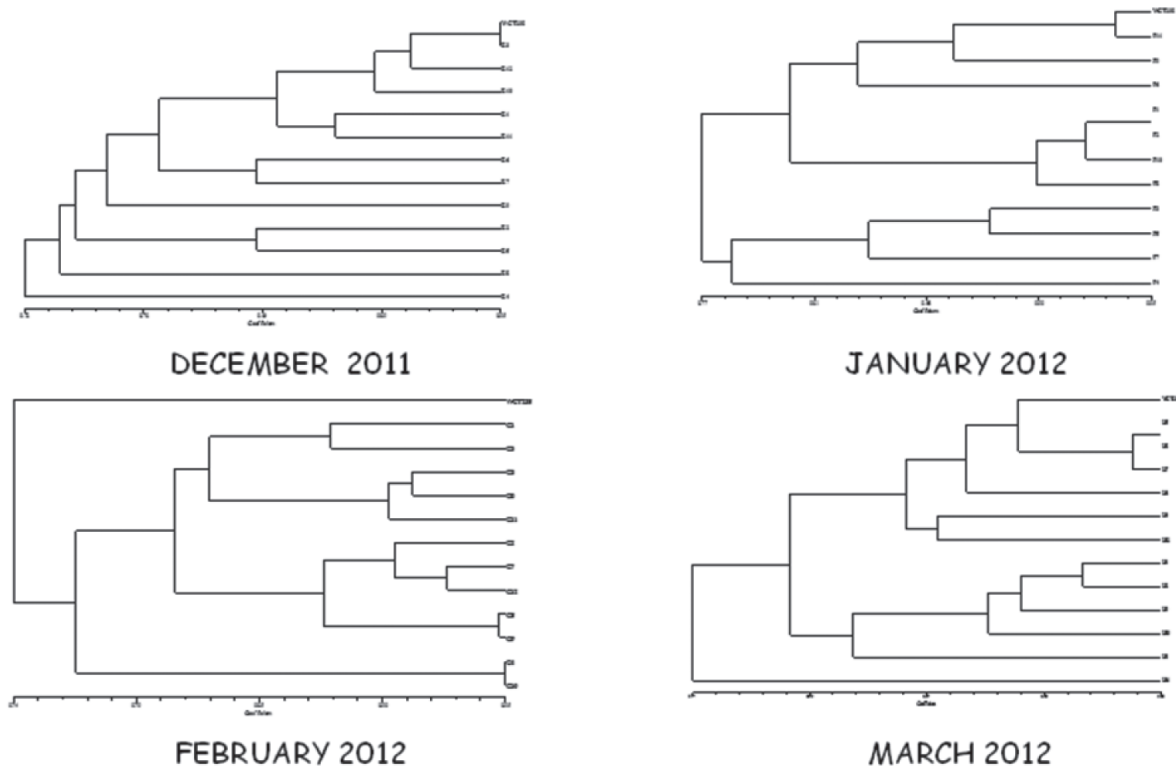
DECEMBER 2011

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FEBRUARY 2012

MARCH 2012

Fig. 2a. UPGMA dendrogram, based on Dice's similarity co-efficient, for mother palm WCT 238 and its OP progenies

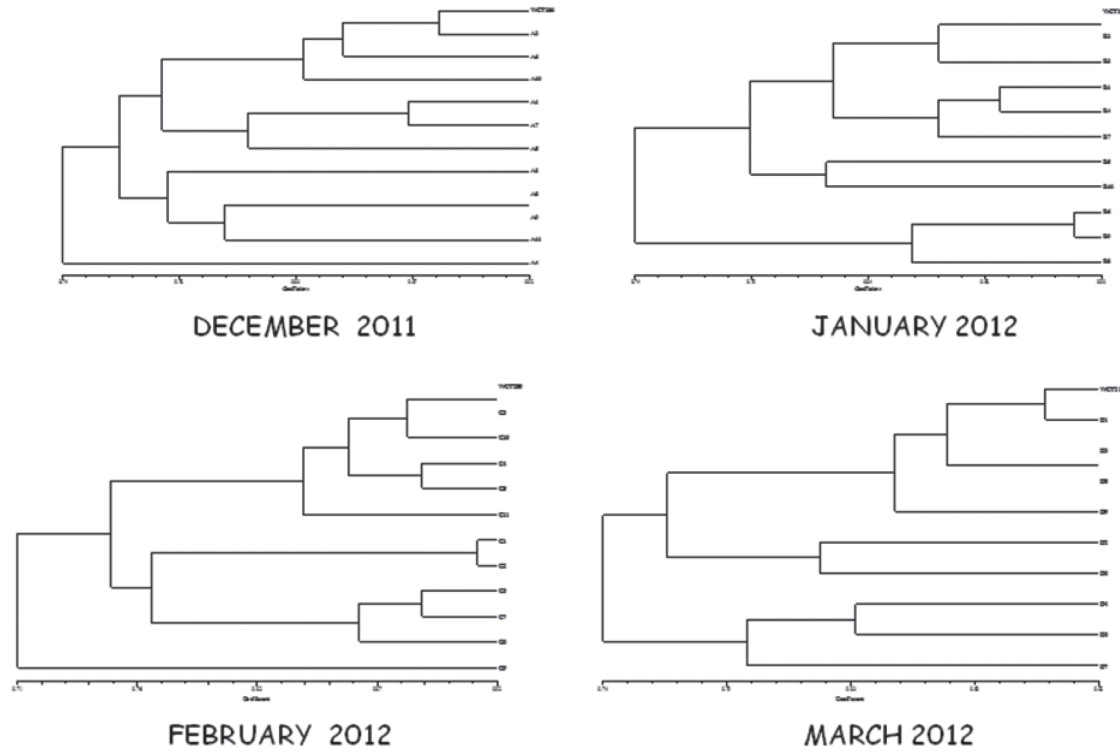DECEMBER 2011

JANUARY 2012

FEBRUARY 2012

MARCH 2012

Fig. 2b. UPGMA dendrogram, based on Dice's similarity co-efficient, for mother palm WCT 238 and its OP progenies

F_{IS} for eight of the loci was below zero, with a mean of -0.0085. Mean F_{ST} (0.25) indicated that the population was highly differentiated. The mean gene flow (N_m), based on mean F_{ST}, was very low (0.749) indicating the absence of extensive gene flow (Table 4).

The genetic structure of plant populations reflects the interactions of various factors, including the long-term evolutionary history of the species (shifts in distribution, habitat fragmentation and population isolation), genetic drift, mating system, gene flow and selection (Schaal *et al*., 1998). The founding number, probability of common origin, kin structure, and inbreeding within populations all have significant effects on genetic differentiation among populations (Whitlock and McCauley, 1990). Differentiation or speciation has mainly occurred during periods when habitats were fragmented (Bridle *et al*., 2004). A high F_{ST} value (0.25) indicated pronounced genetic differentiation among the two studied populations.

In this study, the relatively high genetic differentiation and low levels of gene flow detected

Table 4. Summary of F-statistics and gene flow for the 14 microsatellite loci

Sl. No.	Locus	F_{IS}	F_{IT}	F_{ST}	Nm*
1.	CnCir87	0.1257	0.2314	0.1209	1.8171
2.	CnCirG11	0.1891	0.2931	0.1283	1.6992
3.	CnCirE4	0.1054	0.2533	0.1653	1.2621
4.	CnCirB6	0.2908	0.4694	0.2518	0.7427
5.	CnCirE10	-0.2705	-0.2583	0.0096	25.7897
6.	CnCirB12	0.1226	0.2277	0.1197	1.8387
7.	CnCirC3	0.0276	0.2039	0.1813	1.1288
8.	CnCir56	-0.0213	0.9583	0.9592	0.0106
9.	CnCir74	0.0268	0.1254	0.1013	2.2184
10.	CnCir2	-0.0768	-0.0726	0.0039	64.3889
11.	CNZ26	-0.0892	0.0724	0.1484	1.4351
12.	CNZO4	-0.1801	-0.1458	0.0291	8.3437
13.	CNZO5	-0.2623	-0.1034	0.1259	1.7359
14.	CNZ10	-0.0736	-0.0593	0.0133	18.6008
15.	CNZ17	-0.0229	0.9125	0.9145	0.0234
	Mean	-0.0085	0.2436	0.2500	0.7499

*Nm = Gene flow estimated from Fst = 0.25(1 – Fst)/Fst

283

(Nm = 0.749) strongly indicated that genetic drift had greatly affected the genetic composition of individual populations. In coconut, gene flow between populations is mostly *via* pollen movement. Between-population gene flow was limited by pollen and seed dispersal. Being mainly an insect-pollinated plant, pollen dispersal is limited by the short flight ranges of the insects. Moreover, the limited seed dispersal contributes to the restricted gene flow and increases the probability that individuals in close physical proximity mated with one another. Both effects would promote inter-population differentiation.

Knowledge of genetic diversity within and among the populations is crucial for conservation purposes, when interpreted within a broader ecological and organismic context. Considering the high level of genetic differentiation among populations, preservation of any one population would not protect all the variation in the species. Therefore, several populations including pre-potent and 'super palm' progenies throughout the entire range should be considered for conservation, after molecular analysis.

Results of the Fisher's exact test for Hardy-Weinberg (HW) equilibrium across loci, considering

Table 5. Probability test for departure from Hardy–Weinberg equilibrium (HWE) calculated for the 15 SSR markers

Sl. No.	Locus	P-valve	SE
1	CnCir87	0.8773	0.0038
2	CnCirG11	0.8497	0.0036
3	CnCirE4	0.6926	0.0078
4	CnCirB6	0.9650	0.0016
5	CnCirE10	0.0005 *	0.0002
6	CnCirB12	0.7149	0.0034
7	CnCirC3	0.5924	0.0112
8	CnCir56	0.0000 *	0.0000
9	CnCir74	0.5669	0.0062
10	CnCir2	0.6844	0.0022
11	CNZ26	0.1682	0.0050
12	CNZO4	0.0050	0.0004
13	CNZO5	0.0641	0.0014
14	CNZ10	0.0000 *	0.0000 *
15	CNZ17	0.0000 *	0.0000 *

*P<0.001

heterozygote excess as the alternative hypothesis, showed that only four of the loci had significant (p< 0.001) departures from HW proportions (Table 5).

Genetic diversity within populations

The fixation index ranged from -0.007 (WCT280) to 0.009 (WCT238) with a mean of 0.001, indicating high levels of outcrossing in these populations (Table 6). The mean observed heterozygosity was same as expected, and fixation index (f) was almost equal to zero indicating that the population is randomly mating. Specifically, observed and expected heterozygosity will be the same if the level of external gene flow is the same across all populations.

Table 6. Expected (H_e) and observed (H_o) heterozygosity and fixation index in the two populations

Population	H_e	H_o	f
WCT238	0.325907	0.322843	0.009497
WCT280	0.319978	0.322222	-0.007104
Mean	0.322942	0.322533	0.001287

The locus by locus AMOVA analysis, performed considering among populations and within populations as sources of variation, is given in Table 8. The highest percentage of variation (92%) correspond to the within population component, while the among population component showed low magnitude (8%).

AMOVA revealed that there was a considerable variation between the individuals (60.83%, p< 0.001). Generally, the mating system of flowering plant species greatly affects population genetic differentiation (Hamrick and Godt, 1989).

Mating system analysis

In the case of WCT238, the multi-locus (tm) and single locus (ts) outcrossing estimates for the population were 1.000 ± 0.0 and 0.849 ± 0.0, respectively and the difference between the multi-locus and single locus (tm – ts) was 0.151 ± 0.0. Multi-locus correlation of P (pollen and ovule gene population frequencies) estimate (rpm), single locus correlation of P estimate (rps) and correlation of tm between progeny arrays (rt) were 0.086 ± 0.019,

Table 8. AMOVA analysis for partitioning of SSR variation among and within populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	142.088	1.55919 va	39.43
Among individuals within populations	88	209.862	-0.01038 vb	-0.26
Within individuals	90	216.500	2.40556 vc	60.83
Total	179	568.450	3.95437	

0.098 ± 0.002 and 0.017 ± 0.011, respectively. Difference (rps - rpm) of estimate was 0.012 ± 0.017. The value of the single locus inbreeding coefficient of maternal parents, F, was 0.046 ± 0.021 (Table 9).

In the case of WCT280, the multi-locus (tm) and single locus (ts) outcrossing estimates for the population were 1.0 ± 0.0 and 0.975±0.001, respectively and the difference between the multi-locus and single locus (tm – ts) was 0.025± 0.0. Multi-locus correlation of P (pollen and ovule gene population frequencies) estimate (rpm), single locus correlation of P estimate (rps) and correlation of tm between progeny arrays (rt) were 0.002 ± 0.0, -0.999 ± 0.0 and 0.002± 0.0, respectively. Difference (rps - rpm) of estimate was -1.001 ± 0.0. The value of the single locus inbreeding coefficient of maternal parent, Fm, was 0.0 (Table 9).

In both the populations, both multi-locus and mean single locus (ts) outcrossing rates were relatively high (equal to one), indicating that WCT was allogamous cultivar. The 'protandrous' phenomenon could reasonably account for the high degree of outcrossing in this species. Also, the lack of much difference between the multi-locus (tm) and single locus (ts) estimates indicates that there is no 'biparental inbreeding' in the population. In this study, the absence of 'biparental inbreeding'

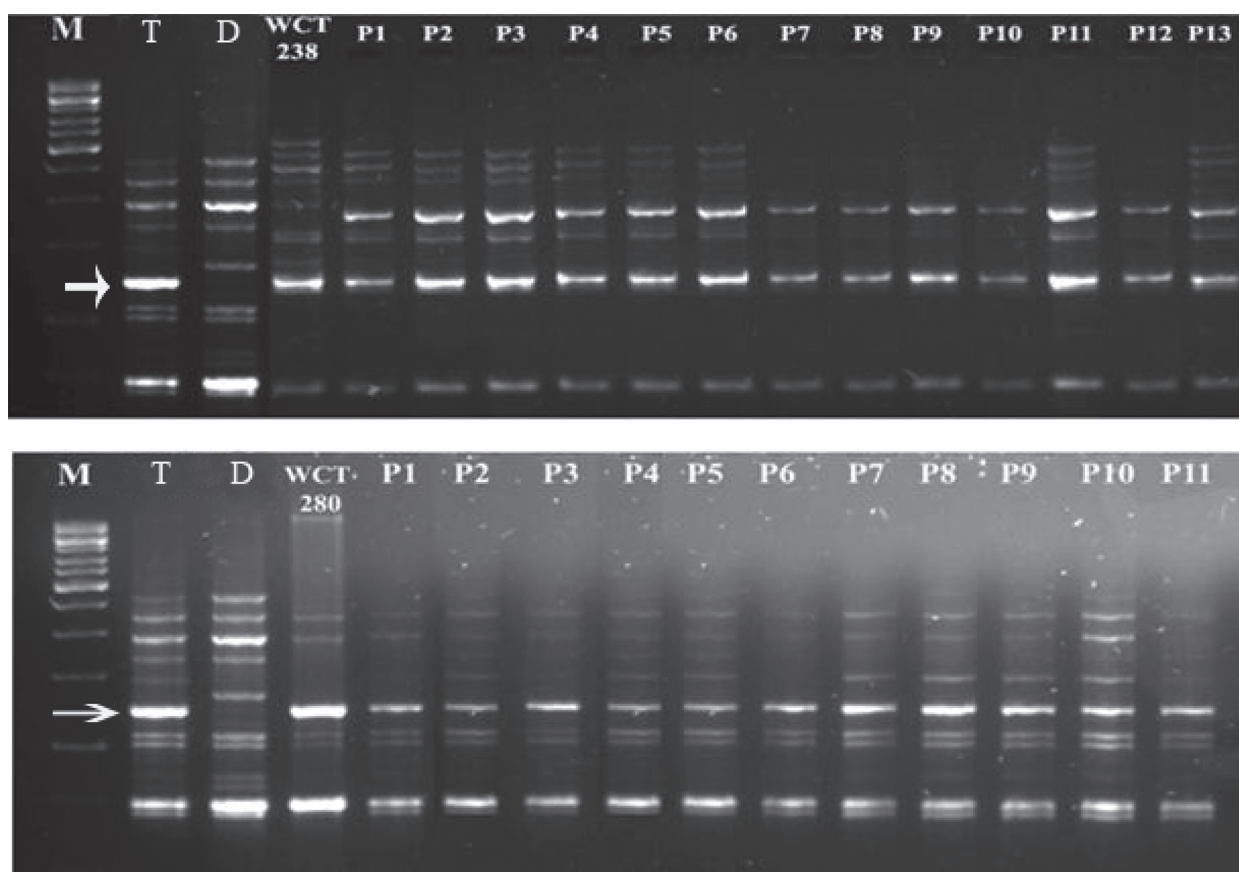strengthens the conclusion that the studied populations were completely outcrossing.

No differences in the outcrossing rates among the mother plants were observed [rt (correlation of tm between progeny arrays) was nil]. The rp (correlation of outcrossed paternity within progeny arrays) values suggest that all the individuals within the population were outcrosses. The rps [single locus correlation of P (pollen and ovule gene population frequencies) estimate] was less than rpm (multilocus correlation of P estimate), which indicated that there was no effect of population substructure on the male similarity between the outcrosses. F (fixation index of maternal parents) was zero suggesting no Hardy–Weinberg deviations exist in the populations, which indicated that individuals within the populations mate at random.

RAPD analysis

The RAPD primer OPBA3 produced a band of around 1200 bp present only in Tall palms and a band of around 1300 bp present only in Dwarf palms (Rajesh *et al*., 2014). The polymorphisms observed between the Tall and Dwarf coconut palms were used as markers for screening the mother palms and their progenies. All the progenies possessed the unique bands of Tall-type palms indicating that the pollen parent in all the cases were Tall palms (Fig. 3 a and b).

Table 9. Estimates of multi-locus (tm), uni-locus (ts), biparental endogamy (tm- ts), and paternity correlation (rp) outcrossing rates and fixation index of maternal generation (Fm) for two populations of WCT. Standard deviations are given in brackets

Family	tm	ts	tm-ts	rpm	rps	rpt	rpm-rps	Fm
WCT238	1.0	0.849	0.15	10.0	-0.999	0.0	-0.999	0.0
	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
WCT280	1.0	0.975	0.025	0.002	-0.999	0.00	-1.001	0.0
	(0.0)	(0.001)	(0.0)	(0.0)	(0.0)	(0.0)	(0.002)	(0.0)

Fig. 3. RAPD profiles of mother palms (WCT 238 and WCT 280) and their progenies (P)
T: Tall Bulk; P: Dwarf Bulk; Arrowhead indicate Tall palm-specific marker

Concluding remarks

Knowledge of genetic diversity within and among the populations is crucial for conservation purposes, when interpreted within a broader ecological and organismic context. Considering the high level of genetic differentiation among coconut populations, preservation of any one population would not protect all the variation in the species. Therefore several populations throughout the entire range should be considered for conservation. If possible, all populations studied should be conserved. The high outcrossing rate and low gene flow among the populations indicated that *ex situ* plantings would result in possible contamination of the different populations.

Another unique and rare phenomenon studied by breeders (Harland, 1957; Ninan and Pankajakshan, 1961; Satyabalan and Jacob Mathew, 1983), is the pre-potency seen among the WCT, which represent rare gene-complexes, exhibiting a high rate of transmission of parental characters to their progenies. This is believed to be controlled by a constellation of genes that cohere but do not recombine even under random mating (Clausen and Hiesey, 1960), thereby preserving for posterity, these rare pre-potent palms showing unusually high yields and stress tolerance. In fact some of these 'super palms' identified in natural populations, with unusually high nut production of over 200 nuts palm^{-1} year^{-1}, even under epiphytotic conditions in hot-spots of the dreaded root (wilt) disease in Central and Southern Kerala, have shown evidence of prepotency as revealed in OP progeny tests conducted at seedling stage (Iyer *et al.*,1979, 1982). This points to the urgent need for screening natural populations of all palm cultivars, both in hot-spots and healthy, disease-free gardens, to identify the rare pre-potent and 'super palms', both by seedling-prediction equations, and precise

molecular mapping as shown in this work, so that they can be conserved in gene banks and utilized in future coconut breeding programmes.

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