

Assessment of genetic diversity in *Calamus vattayila* Renuka (Arecaceae) using ISSR markers

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Calamus vattayila Renuka is one of the medium sized rattans endemic to the Western Ghats of India. We have investigated the genetic variation of three populations of this species using ISSR markers. ISSRs generated a total of 46 markers with 9 primers, of which of 41.3% in Nelliampathy, 39.13% in Kallar and 28.3% in Sholayar were polymorphic fragments with amplicon size ranging from 250 to 2000 bp. The gene diversity was 0.167 in Nelliampathy, 0.163 in Kallar and 0.118 in Sholayar with a total genetic diversity (Ht) of 0.238 among populations. Majority of the genetic diversity was distributed within populations (61.07%) and only (38.93%) was among populations. The percentage of total genetic differentiation (38.3%) among the three populations was relatively higher than the mean reported in many other species. The genetic distance between populations ranged from 0.0794 to 0.2246 and the unweighted pair-group method with arithmetic averaging (UPGMA) cluster analysis and Dollo-parsimony tree, showed the tendency of the individuals to group according to the geographical localities. The genetic differentiation and total gene diversity among the populations of *C. vattayila* were significantly high, indicating the importance for conservation of germplasm representing individuals from all the populations. The result also indicates the possibility of inbreeding in *C. vattayila*.

Key words: Genetic variation, ISSR markers, rattans, Western Ghats

INTRODUCTION

Rattans are spiny climbing palms belonging to the family Arecaceae, often described as 'green gold'. They are exploited for their flexible stems that form the basis of a significant market for cane and cane products. Worldwide, more than 700 million people reportedly trade or use rattan (Sastry, 2000) and INBAR (2012) reported that the international trade in bamboo and rattan amounted to USD 1.9 billion. In India, there are 55 species under four genera (Renuka and Sreekumar, 2012) mainly distributed in the Western Ghats, sub-Himalayan hills, valleys of Eastern and North-eastern India and in Andaman and Nicobar Islands. In recent years, uncontrolled harvesting and deforestation has led to erosion of the genetic diversity and distribution of rattans. Due to overexploitation, alteration of habitat and poor regeneration, rattan resources are declining at an alarming rate and there is an urgent need to initiate resource enhancement of these threatened species (Dransfield, 1979; Evans *et al.*, 2001; Hong *et al.*, 2000). In order to develop scientifically sound, comprehensive and

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resource effective strategy for gene pool conservation, knowledge on the pattern of genetic diversity in space and time is necessary. Molecular markers are widely used for assessing genetic diversity within and among populations. Among molecular markers, ISSR markers (Inter Simple Sequence Repeat) are one of the most useful markers used for investigating genetic variation (Geleta and Bryngelsson, 2009; Pérez de la Torre *et al.*, 2012; Patel *et al.*, 2015), clonal diversity and population genetic structure (Rossetto *et al.*, 1999; Sikdar *et al.*, 2010), cultivar identification (Arnau *et al.*, 2002; Younis, 2008) and phylogenetic analysis (Joshi *et al.*, 2000; Wang *et al.*, 2009; Witono and Kondo, 2006; Suranjana Ray *et al.*, 2010; Haider *et al.*, 2012). ISSR markers has also been used to evaluate genetic diversity within rattans like *Calamus thwaitesii* (Ramesha *et al.*, 2007), and *Daemonorops draco* (Asra *et al.*, 2014).

Calamus vattayila Renuka is one of the solitary rattans endemic to Western Ghats. The species is found sporadically in the evergreen forests between 200-750 m altitudes. This cane is not available in sufficient quantities due to its restricted distribution and unsustainable harvesting due to its solitary habit. In Kerala parts of Western Ghats, three main populations of this species were identified at Nelliampathy, Kallar (Achenkovil) and Sholayar (Sreekumar *et al.*, 2012) forests. These populations are highly fragmented patches mainly due to over exploitation coupled with poor regeneration potential. As a prior step towards initiating the conservation of this species, we have assessed the genetic diversity of this species using ISSR markers.

MATERIALS AND METHODS

Field surveys were conducted to select individuals from three populations (Nelliampathy, Kallar (Achenkovil) and Sholayar) of *C. vattayila* and fresh leaf samples, herbarium specimens and seeds were collected.

ISSR analysis

From the fresh leaf samples collected, total DNA was extracted from 1g of the leaf tissues per plant using the modified CTAB protocol (Doyle and Doyle 1990) and PCR reactions were performed in 25 μ l reaction volumes with 10X Taq buffer with 15mM MgCl₂, 50ng of template DNA, 10mM MgCl₂, 1mM of dNTP mix, 10 picomoles of primer, 2 units of Taq DNA polymerase using PTC 200 Thermal Cycler. The amplification was performed with an initial denaturation of 94^o C for 5 minutes and 30 cycles for 45 seconds of denaturation at 94^o C, 45s annealing (52^oC), and 120s extension (72^oC). The last cycle was followed by incubation for 8 min at 72^o C. Nine ISSR primers were selected out of the 20 primers screened, based on the repeatability of their DNA band profiles and the details of ISSR primers are provided in Table 2. The ISSR products were electrophoresed on 1.5% agarose gel (Sigma, USA) in TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8.0). The gel was visualized under the Electronic UV Transilluminator by Ultra CAM Digital Imaging system.

Data analysis

ISSR products were scored for presence (1) and absence (0) of bands. The data

matrices were analyzed using the Popgene, Version 1.31 package, and a pairwise comparison of populations was made (Yeh *et al.*, 1999). The genetic diversity parameters within populations, viz. number of polymorphic loci and gene diversity were determined. Genetic differentiation between the analyzed populations was calculated according to Nei (1973). The genetic distance matrix was also used to estimate variance components and to test the significance of partitioning of genetic variation (AMOVA; Excoffier *et al.*, 1992) using WINAMOVA. To evaluate the correlation between genetic distance and geographic distance, the product moment correlation coefficients were calculated between the genetic and geographic distance matrices and significance levels of the correlation between these matrices were estimated by mantel test using TFPGA software version 1.3. A phylogenetic tree of all the haplotypes was generated by the Dollo parsimony algorithm method for discrete character data with two states (0 and 1) using PHYLIP Version 3.5 (Felsenstein, 1993) assuming no ancestral states.

RESULTS

Among the different ISSR primers screened from UBC, ISSR series, (Indira *et al.*, 2012) nine ISSR markers (Table 1) were selected for genetic diversity assessment for *C.vattayila*. The percentages of monomorphic and polymorphic bands were 36.9% and 63.1%, respectively; and the number of scorable bands amplified by each primer varied from 6 (UBC 841) to 11 (ISSR 4). The percentage of polymorphic loci (ppl) was observed as 28.3% in Sholayar, 39.13% in Kallar and 41.3% in Nelliampathy. The band size generated by the nine primers ranged from 250 to 2000 bp. On average, effective alleles were 1.28 out of 1.41 observed alleles in Nelliampathy, 1.3 out of 1.39 observed alleles in Kallar and 1.21 out of 1.28 observed alleles in Sholayar. The gene diversity was found to be 0.167 in Nelliampathy, 0.163 in Kallar and lowest in Sholayar with 0.118. Partitioning of variation within and between populations using an analysis of molecular variance (AMOVA) showed that 61.07% of the genetic variability existed as variation within populations ($P < 0.001$; Table 2). The dollo

Table 1: List of ISSR primers selected for the study

Sl. No	ISSR primers	Sequence (5'-3')
1	UBC 834	AGAGAGAGAGAGAGAGYT
2	UBC 835	AGAGAGAGAGAGAGAGYC
3	UBC 841	GAGAGAGAGAGAGAGAYC
4	UBC 855	ACACACACACACACACYT
5	UBC 868	GAAGAAGAAGAAGAAGAA
6	UBC 880	GGAGAGGAGAGGAGA
7	UBC 890	VHVGTTGTGTGTGTGTGT
8	ISSR 4	AAGAAGAAGAAGAAGCC
9	ISSR 5	AGCAGCAGCAGCAGCCA

parsimony tree using PHYLIP clearly indicated the phylogenetic relationships among the populations analyzed (Fig. 2). The UPGMA dendrogram based on Nei's genetic distances showed that Nelliampathy population was more closely related to Kallar (Fig. 3) than Sholayar, even though Sholayar and Nelliampathy are geographically closer. The results show that within population diversity was found to be low in individual populations and an average of Nei's index ($H_s=0.147$) at population level was lower than the mean value ($H=0.22$) of many species (Nybom, 2004). Geographical and genetic distances were not significantly correlated in the mantel test ($r = -.5627; p = 0.3280$).

Table 2: Hierarchical analysis of molecular variance One way (AMOVA)

Variance component	d.f	SSD	MSD	Variation variance	%	P
One region						
Among population	2	56.74	28.37	1.60	38.93%	<0.001
Within population	53	133.71	2.52	2.52	61.07%	<0.001

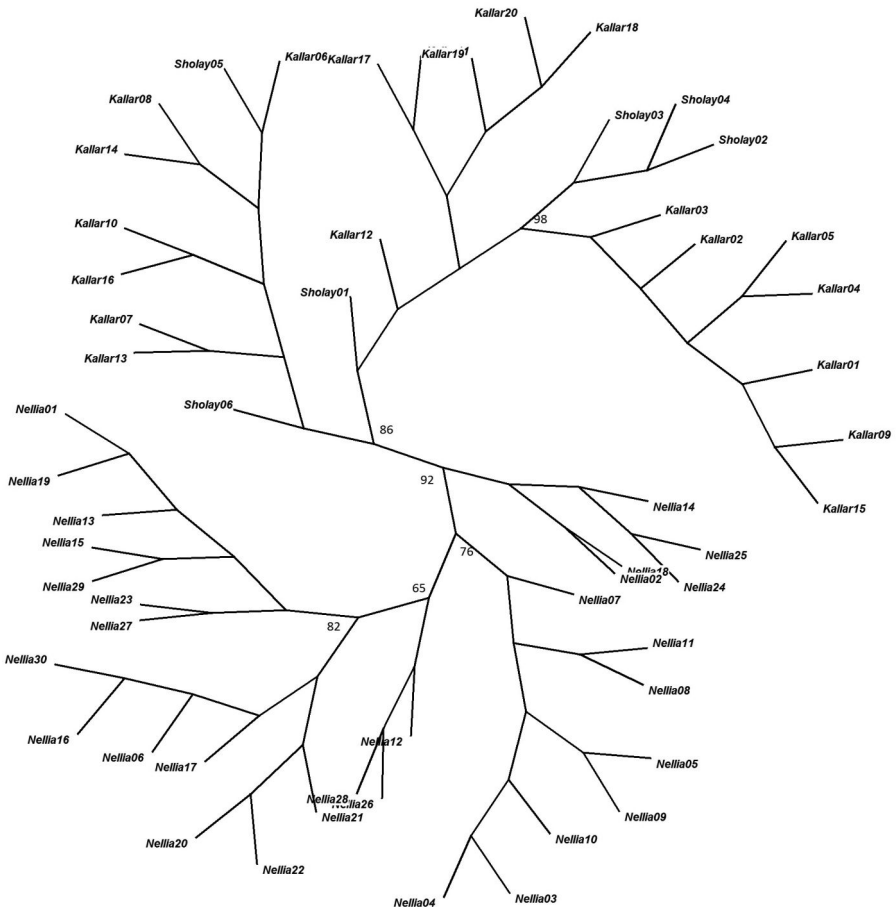


Figure 2: Unrooted Dollo-parsimony phylogenetic tree showing grouping three populations

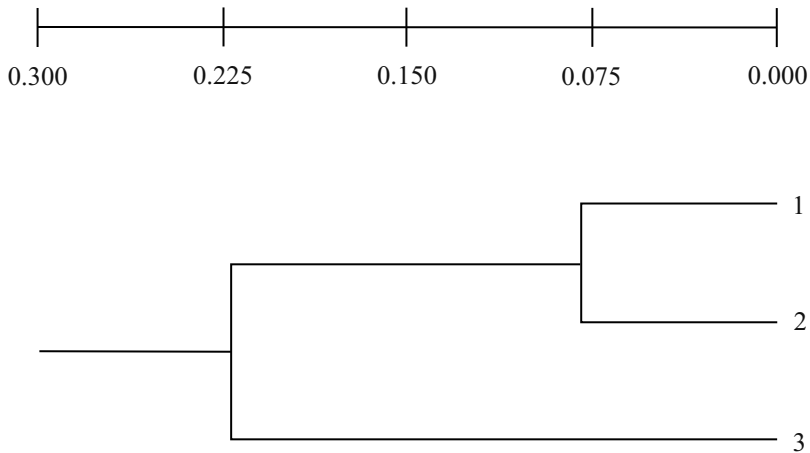


Fig. 3: UPGMA dendrogram of ISSR data based on Nei's genetic distances (1973). (1- Nelliampathy, 2-Kallar, 3-Sholayar)

DISCUSSION

The percentage of polymorphism observed in the present study was 63.1%, similar to earlier reports where high percentage of polymorphism has also been reported in other rattans like *Calamus manan* (66.67-76.67%) in Sumatra, and 70.5% in *Calamus subinermis* in Malaysia (Bon, 1995), *Calamus thwaitesii* ranging from 75% to 91.67% with a mean of 82.77% in 15 Central Western Ghat populations using isozyme markers (Ravikanth *et al.*, 2001) and 95% in *Calamus metzianus* (Sreekumar *et al.*, 2006) using RAPD markers. High levels of polymorphism are indicative of high levels of outcrossing among individuals and between populations (Loveless and Hamrick, 1984). Nei's gene diversity (h) estimate was found to be 0.15, which is very low when compared to other wind pollinated, woody and long-lived tree species in which comparatively much variability within population is reported (Hamrick and Godt, 1990) as in *Populus tremuloides* (0.30) (Yeh *et al.*, 1995) and *Quercus petraea* (0.29) (De Greef *et al.*, 1998). The percentage of total genetic differentiation (38.3%) among the three populations ($G_{st}=0.383$) analyzed using Popgene also revealed that relatively much higher than the mean G_{st} value (0.129) reported in many other species (Hamrick *et al.*, 1992). It is reported that the long-lived, woody and late successional organisms typically represent greater percentage of their variation within populations (Hamrick and Godt, 1990). Similar pattern was observed on *Azadirachta indica* (Kundu 1999) and *Banksia cuneata* (Maguire and Sedgley, 1997). However, studies on *Orobanche cumana* (Gagne *et al.*, 1998) and *Oryza glumaepatula* (Buso *et al.*, 1998) indicated a reverse trend in which most of the genetic diversity was found among populations than within populations. In the present study, the partitioning of genetic variation using AMOVA showed high amount of diversity in intra-populations (61.07%) as reported in long-lived, woody and late successional organisms (Hamrick and Godt, 1990). It is found that the selfing species are generally supposed to allocate most of the genetic variability among populations (Bartish *et al.*, 1999). The most genetically similar populations (Nelliampathy and Kallar) were geographically

separated by a distance of 167 km, while the genetically distant populations (Nelliampathy and Sholayar) were separated by about only 32 km. The test of correlation between the genetic and geographic distance matrices using the mantel test revealed a negative correlation with $r = -.5627$ ($p = 0.3280$), which was supported by the UPGMA dendrogram. At the population level, the species is under continuous threat of degradation; populations are fragmented; remnant forests surrounded by human settlements and are in continuous pressure due to extraction of resources for the furniture industry.

Assessment of genetic diversity parameters of threatened species are an important step towards developing conservation strategies and breeding programmes (Renuka, 1994; Renuka *et al.*, 1998). The genetic differentiation and total gene diversity among populations were high, indicating possibility of restricted gene flow or inbreeding in *C. vattayila* populations. To ensure sufficient quantities of seeds, seed stands were established representing three populations at Malayattur forests and also introduced at germplasm of Kerala Forest Research Institute.

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