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Research article

Assessment of genetic diversity in natural populations of *Calamus guruba* Buch.-Ham. ex Mart. using ISSR marker

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Abstract: Rattan palms are one of the important nontimber forest produce which forms an integral part of the tribal population and contribute significantly in the rural economy. The demand of the rattan resources is substantial but most of the raw material is being collected from natural stands; and therefore, there is tremendous pressure on natural populations. *Calamus guruba* is one of the important rattan species of India and extensively harvested for a wide range of end products. In the present study, three populations of *C. guruba* naturally growing in Mizoram were characterized for their genetic diversity and population structure using Inter-Simple Sequence Repeat (ISSR) markers. A total of 197 bands generated in PCR amplification of 67 individuals with 10 ISSR primers. Among all the primers UBC 808 displayed maximum 25, while UBC 807 and UBC 827 showed the lowest 13 numbers of bands. Relatively high genetic diversity (*PPB* = 98.98 %, *h* = 0.271, *I* = 0.341) was recorded for the species with a moderate level of genetic differentiation (*PhiPT* = 0.282). Analysis of molecular variance revealed that the genetic differentiation among the populations is the significant and large proportion of the genetic variation (72%) resides among the individuals within a population, whereas only 28 % were found among the populations. UPGMA tree, PCoA plot and STRUCTURE analysis revealed that two ancestral groups captured the entire genetic variability. The baseline study on *C. guruba* is of paramount importance in devising programs for conservation and improvement.

Keywords: *Calamus guruba* - Genetic diversity - Genetic differentiation - ISSR - Rattan.

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INTRODUCTION

Rattans are climbing spiny palms known for their lightness, strength, durability and elasticity. About 650 species of rattans have been reported in the world under 22 genera and around 400 of them belong to the genus *Calamus* (Govaerts *et al*. 2014). *Calamus* genera comprising most of the commercial rattan species; is predominantly distributed in Asia from the Indian subcontinent, China, Malaysia, Indonesia, Fiji to tropical and subtropical parts of eastern Australia. India is a home of about 60 species belonging to 4 genera and mainly distributed in three hotspots *viz.*, Peninsular India (Western Ghats), North East India and Andaman and Nicobar Islands (Ravikanth *et al*. 2001, Umashaanker *et al*. 2004). Throughout their natural range, rattan species are found in a wide variety of forest and soil types from alluvial plains to moist hill forest up to 2000 m elevation (Renuka 2002).

Rattans are harvested by local communities either for subsistence or commercial utilization and contribute significantly to the rural economy (Ravikanth *et al*. 2001). Many small and large scale industries of furniture, cottage, handicraft, basketry and sports equipment are solely dependent on rattan for their raw material. With the increasing preference of cane products; the demand for raw material has been significantly increased locally as

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well as globally, which results in heavy extraction from the natural forest (Senthilkumar *et al*. 2014). Continuous and unscientific extraction of rattans along with changes in land use patterns like shifting cultivation in North East India has reportedly threatened several economically important rattan species (Thomas *et al*. 1998, Singh *et al*. 2004, Raj *et al*. 2014). A significant change has been recorded in the distribution of rattan palms over the years that might be attributed to changes in land use pattern, shrinkage of the natural forest cover, selective exploitation of stems for the furniture and handicrafts, poor natural regeneration etc. (Renuka 2002).

Natural variation is the key requirement for selection of superior genotypes and adaptation towards climate change; hence, conservation of available genetic resources needs to be accorded the highest priority (Rao & Rao 2000). Genetic variability exists in the base populations bestow them the ability to survive in long-term by accommodating new selection pressure brought by environmental changes and serves as a resource for tree breeding and improvement programs (Frankham 2008, Porth & El-Kassaby 2014). Every genetic improvement programme is based on the assumption that the species has enough genetic variability for the traits of interest that could be exploited during domestication and selection (Renuka *et al*. 1998). Despite of the growing importance and pressure over natural stands of rattans, no serious effort was made to characterize the genetic variability existed among the natural populations. Knowledgebase for the genetic diversity, population structure and spatial patterns of genetic variation are highly useful in devising sound conservation plan of the species. As per the India State of Forest Report, the total forest cover of North East India has been declined significantly (FSI 2017). Therefore, the concern over the state of genetic resources is paramount, particularly for the species endemic to the region. Despite of the wide distribution of rattans, most species are endemic. In India, three geographical regions have specific rattan flora with minimum overlapping distribution pattern (Renuka 2002). However, unlike the attention received by rattans of the Western Ghats in peninsular India, only limited efforts were made in addressing the conservation of rattans of North East India (Lyngdoh *et al*. 2005). Since rattans are distributed in small pockets and knowledge base for their genetic structure and diversity is only poorly developed, *in situ* conservation sites for conservation are yet to be established (Rawat & Ginwal 2009). Therefore there is an instant need to generate baseline data of natural populations for scientific management and conservation of this valuable depleting resource for future, along with the cultivation of commercially important species.

Calamus guruba Buch.-Ham. ex Mart. (Family: Arecaceae) locally referred to as 'Thilte' in Mizoram, is one of the most important dioecious species of rattan found in Southeast Asia viz. Malaysia, Indonesia, Borneo and the Philippines. In the Indian subcontinent, it occurs in the natural forest areas of Odisha, Chhattisgarh, coastal districts of Andhra Pradesh and lower Western Ghats in the states of Tamil Nadu and Kerala (Sinha *et al*. 2017). However, the species also reported to exist in dense tropical and lower subtropical forests of North East India (http://indiabiodiversity.org/species/show/258795). *C. guruba* is extensively harvested by forest-dwelling communities for making a wide range of furniture products and handicraft articles Due to its advantages of the high-quality flexible canes for furniture manufacturing and the tender shoots as food items; it is one of the most economically important rattan species of India (Mahzuz *et al*. 2013). Despite of its enormous usages, the regeneration potential of wild populations of *C. guruba* has been declining gradually due to several biotic and abiotic factors (Sinha *et al*. 2017). Some populations of Odisha have been genetically characterized using Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers (Sinha & Panda 2016). In the present study, three populations of *C. guruba* were explored in Mizoram and characterized for their genetic diversity and population structure.

MATERIALS AND METHODS

Plant Materials

Leaf samples of 67 genotypes representing 3 populations were collected along with geospatial parameters using GARMIN 650 model of Global Positioning System (GPS) during 2015–17. Two populations (CG01 and CG02) were located in Zongaw Reserve Forest, an un-notified Community Reserve Forest of Mizoram while another population was present in Bungthlang forest of Mizoram. The map of the sampled populations in the distribution range of *C. guruba* was prepared using the software Arc-GIS ver 9.2 (Fig. 1). Two sampled genotypes were at least 100 m apart at each site. Fresh leaves were preserved during long field tours by desiccating with silica gel and desiccated samples were brought to the laboratory of Genetics and Tree Propagation Division, Forest Research Institute, Dehradun for further genotyping work.

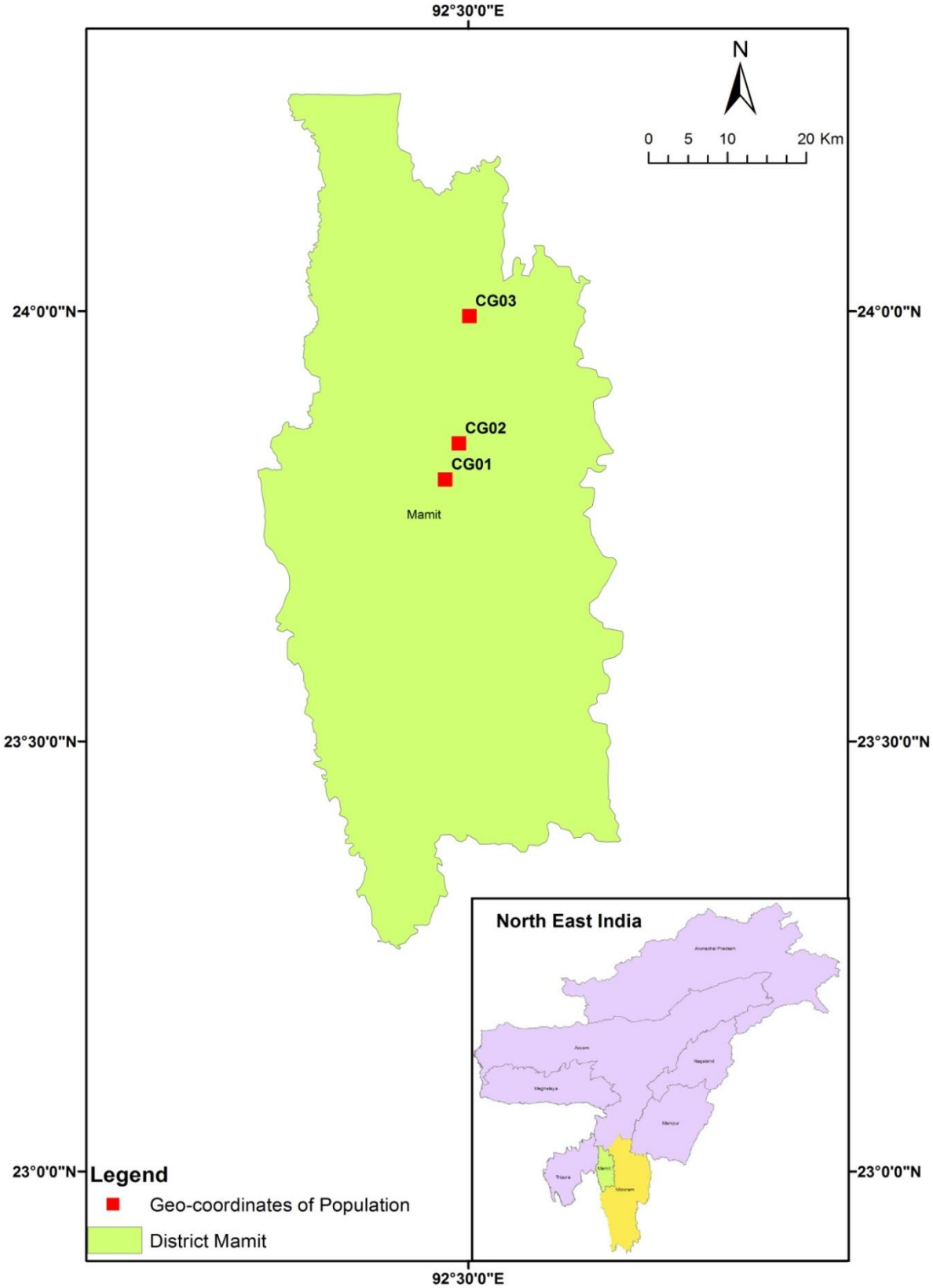

Figure 1. Distribution map of sampled populations of *Calamus guruba* Buch.-Ham. ex Mart.in Mizoram.

Genomic DNA Extraction

Genomic DNA was extracted using standard protocols of Doyle & Doyle (1987) with minor modifications. Frozen tissues were grounded in liquid nitrogen and incubated at 60ºC for 60 minutes in pre-heated CTAB extraction buffer followed by mixing with chloroform-isoamyl alcohol (24:1). The supernatant was pipetted out after centrifugation and precipitated with equal volume of chilled isopropanol overnight. The precipitated DNA was first washed with a solution containing 96% ethanol and 0.3 M Sodium acetate followed by washing with

70% ethanol. After vacuum drying, DNA pellet was re-suspended in 100 µl of 10 mMTris-EDTA buffer (pH 8.0). Qualitative and quantitative analysis of genomic DNA was carried out using 0.8% agarose gel and Biophotometer (Eppendorf), respectively and diluted for final working concentration of 30 ng μl^{-1}.

PCR amplification with ISSR primers

Ten ISSR primers (Table 1) showing positive amplification in *C. guruba* were selected in the initial screening and the annealing temperature was optimized through gradient PCR for clear and distinct banding pattern. Each sample was subjected to the PCR amplification with the 20 µl PCR reaction mixture containing 60 ng of template DNA, 2 µl of 10x PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primer, 0.6 units of *Taq* DNA polymerase and 12.98 µl nuclease free water. The cycling conditions included an initial denaturation at 94°C for 5 min; then 40 cycles of 94°C for 30 Sec, 54–57.5°C for 30 Sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were electrophoretically separated in 2% agarose gel buffered with 1x TBE. A 100 bp DNA ladder was used as a size marker. After staining with ethidium bromide (0.5 µg ml^{-1}), the DNA fragments were visualized and captured using a gel documentation system.

Table 1. Details of ISSR primers used for genotyping.

Primers	Sequence (5' - 3')	Optimized annealing temperature (°C)
PCP-1	GACGACGACGACGAC	55.8
PCP-2	AGGAGGAGGAGGAGGAGG	55.0
PCP-3	GTGCGTGCGTGCGTGC	54.6
UBC 807	AGA GAG AGA GAG AGA GT	57.5
UBC 808	AGAGAGAGAGAGAGAGC	57.5
UBC 826	ACA CAC ACA CAC ACA CC	54.5
UBC 827	ACA CAC ACA CAC ACA CG	54.5
UBC 841	GAG AGA GAG AGA GAG AYC	55.4
UBC 888	BDB CAC ACA CAC ACA CA	56.2
UBC 890	VHV GTG TGT GTG TGT GT	54.5

Data Analysis

Each band was considered as a locus with two alternative alleles and scored manually as 1 (presence) and 0 (absence) in a binary matrix. Input files in different formats were prepared as per the requirement of different software. The parameters like observed number of alleles (*Na*), effective number of alleles (*Ne*), percentage of polymorphic bands (*PPB*), allele frequency, number of private bands (*PB*), Nei's gene diversity (*h*) and Shannon's Information index (*I*) were calculated to describe the statistics of genic variation for all the loci at population and species level using software *POPGENE* ver 1.32 (Yeh *et al*. 1999) and *GenAlex* ver 6.5 (Peakall & Smouse 2012). Total genetic diversity (H_T) and proportional variance resided within the populations (H_S) were also calculated in subdivided populations through *POPGENE*. Analysis of molecular variance (AMOVA) was performed using software *GenAlEx*, which allow the partitioning of genetic variation among the populations. The genetic differentiation between populations was determined using phiPT value (an analogue of F_{ST}) that allows within-population variance to be suppressed and simply calculate population differentiation based on the genotypic variance in binary data. For interpretation, another measure of genetic differentiation (G_{ST}) was also calculated through *POPGENE*. The amount of gene flow (*Nm*) among populations is a measure of the effective number of migrants per population per generation. It was estimated indirectly from the G_{ST} values at each locus and from the average values over all loci by applying McDermott & McDonald's (1993) formula [$N_m = 0.5(1-G_{ST})/G_{ST}$] for the studied populations using software *POPGENE*.

Genetic relationship among the genotypes was studied by constructing a dendrogram based on pair-wise genetic distances among the individual genotypes calculated through Jaccard similarity coefficient using Unweighted Pair-Group Method with Arithmetic averages (UPGMA) in *NTSYSpc* ver 2.02 (Rohlf 1998). To check the consistency of the clusters obtained in UPGMA dendrogram, Principal coordinate analysis (PCoA) was performed using *GenALEx*.

The Bayesian model-based clustering method was used to elucidate the genetic structure of the populations using *STRUCTURE* software ver 2.2 (Pritchard *et al*. 2000). Ancestry model with admixture under the assumption of correlated allele frequencies was used to determine the posterior probability. Simulations were run 10 times for each value of K (1 to 4) with 50,000 Markov Chain Monte Carlo (MCMC) sampling runs after a burn-in period of 50,000 iterations.

RESULTS

Gene diversity and genetic differentiation

PCR amplification with 10 ISSR primers generated 197 bands in 67 individuals representing 3 different populations of *C. guruba*. Figure 2 presents the polymorphic banding pattern generated by ISSR primer PCP1 in populations of *C. guruba*. Among all the primers UBC 808 displayed maximum 25, while UBC 807 and UBC 827 showed the lowest 13 numbers of bands. High genetic variability was revealed by the percentage of polymorphic bands (PPB) recorded at species (98.98 %) and population level (63.45%). The average percentage of polymorphic bands (PPB) among the populations was ranged from 52.28% (CG02) to 70.56% (CG03). Mean genetic diversity was estimated by calculating Shannon's Information indices (*I*) which were recorded as 0.271 for population and 0.341 for species. Among studied populations, the highest degree of variability was recorded for the population of Bungthlang forest, CG03 (*h* = 0.191 and *I* = 0.293) and lowest for a population of Zongaw Reserve Forest, CG02 (*h* = 0.162 and *I* = 0.246) (Table 2). As per the Nei's analysis of gene diversity in subdivided populations, total genetic diversity (H_T) of the species was recorded as 0.215, out of which 81.65% (0.176) was found to reside within the population (H_S).

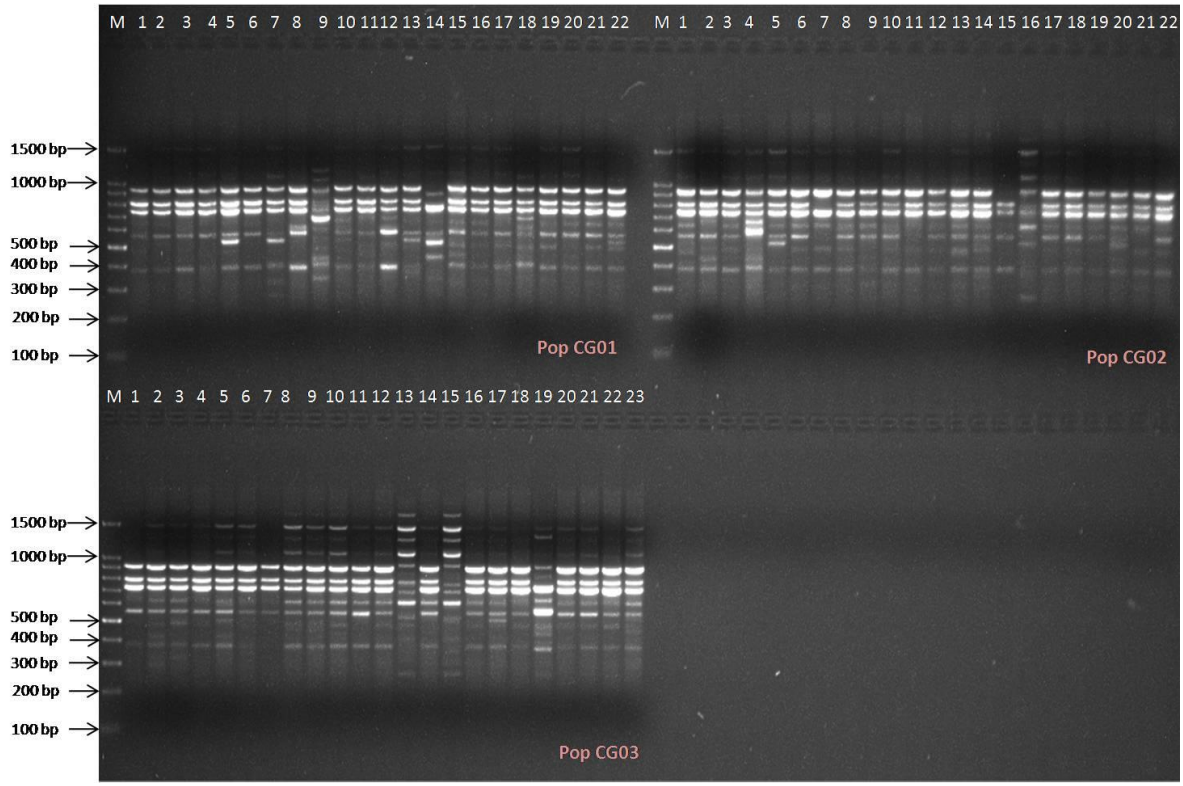

Figure 2. Band profile generated by ISSR primer PCP1 in 3 populations of *Calamus guruba* Buch.-Ham. ex Mart. Lane M represents 100bp DNA ladder; Lane 1, 2,3……n represent "n" samples of the respective populations.

Table 2. Gene diversityover loci for each sampled population of *Calamus guruba* Buch.-Ham. ex Mart.

Population ID	No. of Polymorphic Bands	Percentage of Polymorphic Bands (*PPB*)	No. of Private Bands (*PB*)	Nei's gene diversity (*h*)	Shannon's Information Index (*I*)
				Mean (SD)	Mean (SD)
CG01	133	67.51	25	0.175 (0.180)	0.273 (0.256)
CG02	103	52.28	7	0.162 (0.194)	0.246 (0.277)
CG03	139	70.56	34	0.191 (0.194)	0.293 (0.271)
Mean of Populations	125	63.45	-	0.176 (0.014)	0.271 (0.024)
Overall for Species	195	98.98	-	0.216 (0.178)	0.341 (0.239)

Analysis of molecular variance (AMOVA) showed 72% of the variation existed within the populations and 28% among the populations (Table 3). Variance estimates were based on 999 permutations. Accordingly, the difference between the individuals within the populations was statistically significant with P value <0.001. High values obtained for the estimated measures of genetic differentiation (G_{ST} = 0.184 and *PhiPT* = 0.282) indicates

the moderate level of genetic differentiation among the populations, that was further supported by the presence of a large number of private bands (bands unique to a single population). A total of 66 private bands were detected, of which 34 (51.52%) were present in the population of Bungthlang forest (CG03) while rest of the 32 (48.48%) were present in the other two populations from Zongaw Reserve Forest (CG01 and CG02). Nei's measures of genetic distance were also estimated among the populations which ranges from 0.034 (CG01 and CG02) to 0.103 (CG01and CG03) (Table 4).

Table 3. Analysis of molecular variance (AMOVA) for populations of *Calamus guruba* Buch.-Ham. ex Mart..

Source of variation	DF	SS	MS	Est. Var.	% var.	GD	*P* (rand >= data)
Among populations	2	379.30	189.65	7.63	28	*PhiPT*= 0.282	0.001
Within populations	64	1240.40	19.38	19.38	72		
Total	66	1619.70		27.01	100		

Note: DF, Degree of freedom; SS, Sum of Square, MS, Mean Sum of Square; Est. Var.,Estimated variance; % var.,Percentage of variation; GD, Genetic differentiation; *P*(rand >= data), Probability for *PhiPT* is based on standard permutation across the full data set.

Table 4. Pair-wise Nei's genetic distance and genetic identity among populations.

	CG01	CG02	CG03
CG01	0.000	0.9667	0.902
CG02	0.0339	0.000	0.9149
CG03	0.1031	0.089	0.000

Note: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Genetic structure and relationship among the populations

The UPGMA tree revealed that genotypes of both the populations of Zongaw Reserve Forest (CG01 and CG02) were found to be genetically similar and clustered together which later separated into different sub-cluster of respective populations (Fig. 3). Genotypes of the population of Bungthlang forest (CG03) constitute another major cluster which was genetically dissimilar from others. Moreover, some genotypes were not included in any major cluster and remained as an outlier. Clustering pattern resulted from UPGMA dendrogram was also supported by principal coordinate analysis (PCoA). The PCoA plot showed that the first principal coordinate accounts for 19.49 % of the total variation and separates population of Bungthlang forest (CG03) from other two populations of Zongaw Reserve Forest (CG01 and CG02). Second principal coordinate accounted for only 8.07 % of the variation which couldn't able to separate any of these populations (Fig. 4).

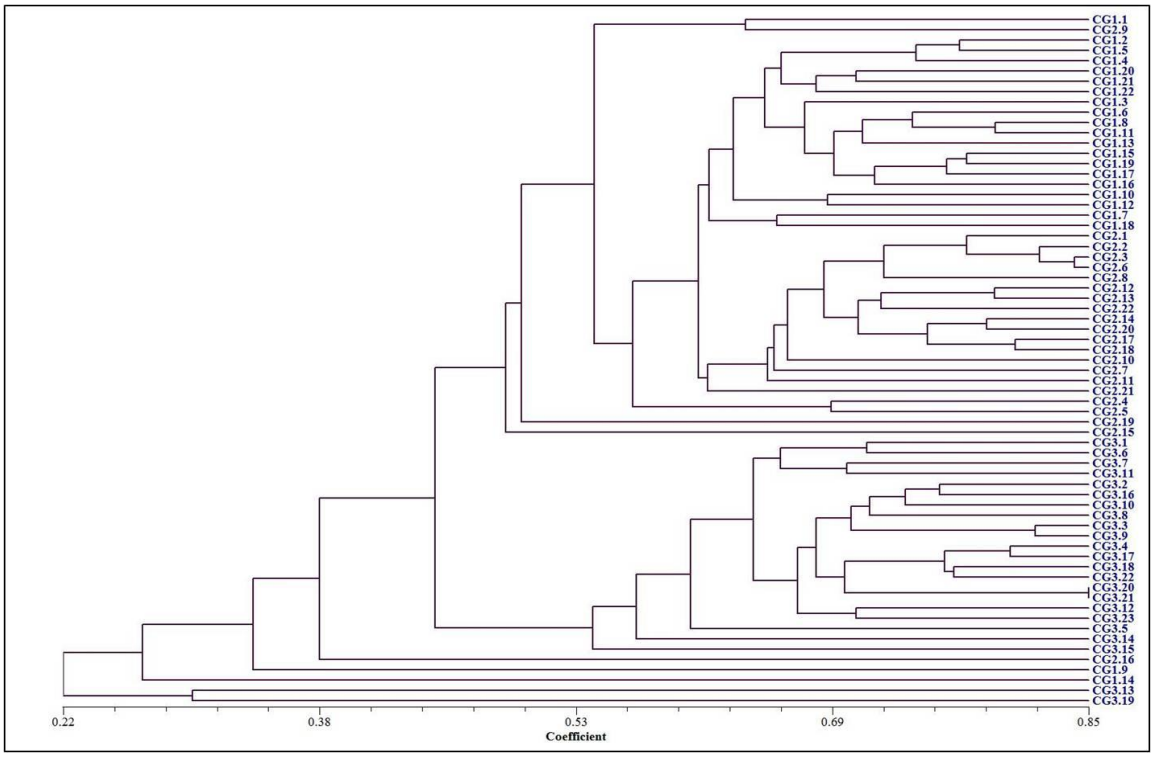

Figure 3. Dendrogram based on genetic distances between individual genotypes obtained from ISSR markers using UPGMA algorithm. Individual genotypes were labelled as decimal points of each population number like CG1.1 to CG1.22 represent samples of population CG01.

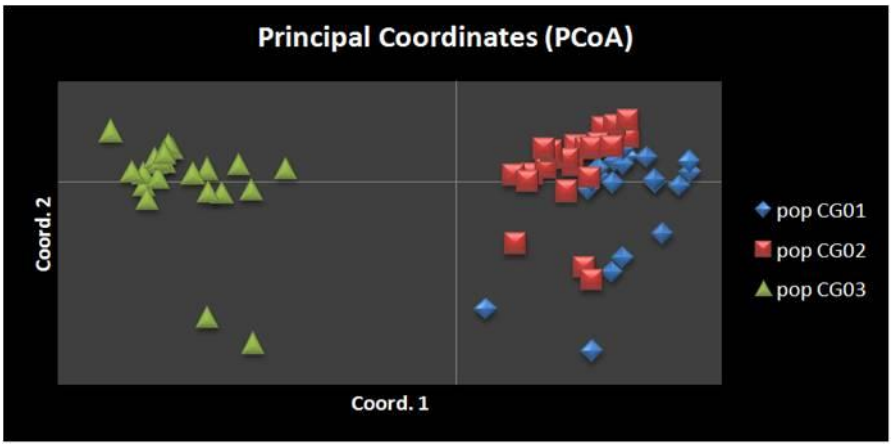

Figure 4. Spatial distribution of the genotypes of *Calamus guruba* Buch.-Ham. ex Mart. in the 2-dimensional plot of Principal Coordinates Analysis (PCoA) based on genetic distances.

Bayesian clustering method was used to elucidate the genetic structure of the sampled populations. A posterior probability [Estimated Ln Probability of Data, LnP(D)] was calculated for each K value. The K value provides the maximum likelihood, called LnP(D) in STRUCTURE, is generally considered as the optimal number of subdivisions but the distribution of LnP(D) values create a plateau in the plot (Fig. 5A). Thus, another *ad hoc* quantity (ΔK) was used to overcome the difficulty for interpreting the real K values using the graphical method suggested by Evanno *et al*. (2005). The highest value of ΔK with a clear peak was obtained for K=2 and hence it was considered that two ancestral groups captured the entire variability among the sampled genotypes (Fig. 5B). As per the inferred ancestries (Q matrix) of the population determined for K=2, all the genotypes of 3 populations were clearly defined by 2 clusters with proportional membership coefficient of more than 0.9. Clustering pattern was in accordance to the UPGMA dendrogram i.e. two populations of Zongaw Reserve Forest (CG01 and CG02) were defined in a single cluster while another population of Bungthlang forest (CG03) was defined in a separate cluster. F_{ST} values for clusters 1 and 2 were recorded as 0.365 and 0.253, respectively. The estimated membership coefficients of the analyzed individuals in each group were represented as a bar plot (Fig. 5C). Each individual of the sample is represented by coloured bars separated by vertical line divided into K coloured segments with the length of each segment being proportional to the estimated membership in each of the K inferred groups. On, X-axis of bar plot, the numbers 1–67 represents individual genotypes of the 3 corresponding populations as depicted in the brackets.

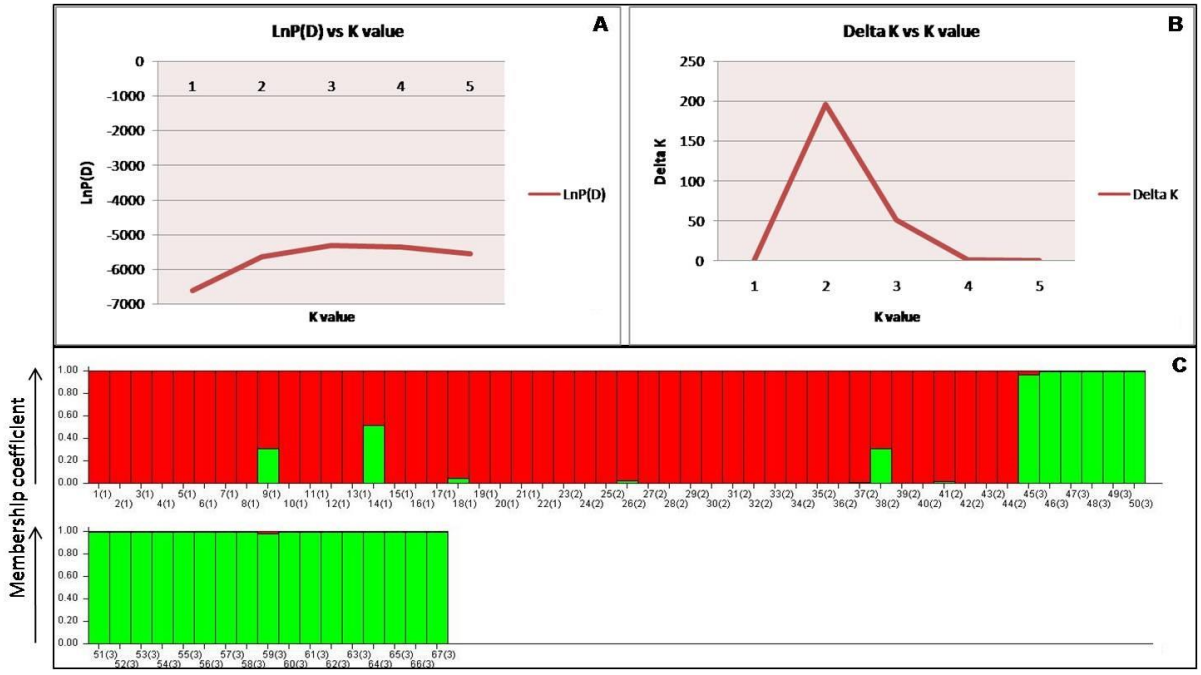

Figure 5. Graphic representation of the estimated probability of data for each K value and Bar plot for estimated individual Q-matrix at optimum K: **A**, Plot of Bayesian posterior probability of data [LnP(D)] with increasing K; **B**, Magnitude of ΔK as a function of K; **C**, Bar plot for estimated individual Q-matrix at K=2 for the genotypes of *Calamus guruba* Buch.-Ham. ex Mart.

DISCUSSION

Three natural populations of *Calamus guruba*, a commercially important rattan palm of North East India were characterized for the genetic diversity and population structure. High level of genetic diversity has been recorded for the species (*PPB* = 98.98 %, *h* = 0.216, *I* = 0.341) with moderate level of genetic differentiation (G_{ST} = 0.184 and *PhiPT* = 0.282) as compare to other rattan species *viz.*, *Calamus palustris* var. *malaccensis* Becc. (*h* = 0.153) in Peninsular Malaysia (Sitisalwana *et al*. 1998), *C. flagellum* Griff. ex Mart. (*h* = 0.201) in Arunachal Pradesh (Lyngdoh *et al*. 2005), *C. thwaitesii* Becc. (*h* = 0.272–0.333) in central Western Ghats (Ramesha *et al*. 2007) and *C. vattayila* Renuka (*h* = 0.150) in Kerala parts of Western Ghats (Priya *et al*. 2016). Low genetic diversity with very high genetic differentiation (*I* = 0.146 and G_{ST} = 0.726) was reported in the Indonesian populations of *Daemonorops draco* due to geographical isolation and apomixtic behaviour of the species (Asra *et al*. 2014).

Generally, species with a wide geographical range tends to maintain a high level of genetic diversity than that of geographically localized species (Hamrick & Godt 1989). Similarly, outcrossing species tend to be genetically diverse in respect to self-pollinated species (Hamrick & Godt 1996). Based on this assumption, a high level of genetic diversity was expected in rattan palms because of their wide range of distribution as well as outcrossing behaviour due to dioecious nature of flowering (Renuka *et al*. 1998). In the present investigation, genetic differentiation (G_{ST} = 0.184) of populations were found to be comparable to the mean value of other out-crossing species (G_{ST} = 0.23); but relatively lower than the mean value of the widely distributed species (G_{ST} = 0.33) (Nybom & Bartish 2000). Although, the Rattans are widely distributed in India; but most of the species are endemic to specific geographical regions (Renuka 2002). Lower genetic differentiation recorded in *C. guruba* may be attributed to its restricted distribution.

Clustering pattern showed two ancestral groups comprising entire variability and most of the genotypes were clustered in accordance of their geographical distribution *i.e.* genotypes belong to a particular geographical area were clustered together. Therefore it can infer that both the populations of Zongaw Reserve Forest are actually single major population and share common ancestral gene pool but quite different from another population of Bungthlang forest. Presence of a large number of private bands and high value of differentiation measures depicted in natural populations indicates that considerable genetic changes adopted by populations in response to habitat fragmentation, genetic drift, and/or barriers to gene flow. The G_{ST} value of 0.184 recorded in *C. guruba* will generally be considered as moderate differentiation and indicates that the structuring between subpopulations is fairly good (De Vicente 2004).

The present investigation revealed that sufficient genetic diversity exists in the sampled populations of the *C. guruba* but the populations of both regions are significantly different from each other comprising various unique alleles. Therefore, conservation measures are required to protect the rattan biodiversity in its natural habitat by preserving rare alleles. The basic but utterly useful information generated in our study would be highly beneficial in devising conservation, management and genetic improvement programme for the depleting rattan genetic resources.

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