J. For. Res. (2020) 31(5):1899–1911
https://doi.org/10.1007/s11676-019-00984-z

ORIGINAL PAPER

Intraspecific genetic variability, differentiation and evolutionary relationships revealed through microsatellite loci in seven economically important *Calamus* species

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Received: 15 May 2018 / Accepted: 28 June 2018 / Published online: 5 June 2019
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Abstract Population density, species richness and critical population parameters are crucial in determining the levels of gene diversity in dioecious species of the genus *Calamus.* The extent of intraspecific and intrageneric genetic variability in *Calamus* from the southern Western Ghats of India was studied using 26 microsatellite markers by sampling 227 individuals belonging to seven economically important species. The heterozygosity of microsatellite loci ranged from zero to 0.78. Average gene diversity within species was 0.13; in all species it was 0.18 and amongst species was 0.06. The Shannon Information Index was the lowest for *Calamus metzianus* (0.11), whereas it ranged from 0.16 to 0.26 for other species. The expected heterozygosity varied from 0.08 to 0.18. *Calamus hookerianus* and *Calamu travancoricus* showed the highest genetic differentiation (44%) revealed through *Fst* values, whereas the lowest (22%) was observed between *Calamus gamblei* and *Calamu thwaitesii.* Population structuring and phylogenetic analysis differentiated the seven species. Due to overexploitation and loss of rare alleles, small populations could lead to fertilization between closely related individuals, resulting in inbreeding and increasing the risk of extinction. This could be important for species such as *C. metzianus* where allelic polymorphism was 23%, whereas for all other species it was 38% to 46%. Genetic diversity "micro-hotspots" were identified from the protected area network of the southern and central Western Ghats with highest observed heterozygosity. Four micro-hotspots from the Agasthyamalai Biosphere Reserve and the Pushpagiri Wildlife Sanctuary may be possible for long-term conservation programs. The findings of this study lay a strong foundation for strengthening protected area networks, especially areas with intermediate levels of disturbance.

Keywords Genetic diversity · Differentiation · Population structure · Conservation · Microsatellites · *Calamus* spp.

Project funding: The work was supported by the Department of Biotechnology, Government of India (Order No. BT/156/NE/TBP/2011) and the Kerala State Council for Science, Technology and Environment (KSCSTE), Thiruvananthapuram (Order No. 234/KBC/2012/KSCSTE).

The online version is available at http://www.springerlink.com.

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Introduction

Rattans are spiny climbing palms belong to the family Arecaceae and consist of some 600 species in 14 genera. *Calamus* is the most widespread with 370 species occurring in both tropical and subtropical regions of Africa and south-east Asia (Uhl and Dransfield 1987). In peninsular India, the genus is represented by 22 species (Anto et al. 2001), and recently several nomenclatural changes have been proposed for the Indian species (Sreekumar and Henderson 2014). Over-exploitation of rattan has considerably depleted natural populations, particularly in the southern regions of India, and consequently raw materials

are being collected from the unexplored north-eastern states of the country (Raj et al. 2014). [In fact, rattan furniture industry is growing at US$4 billion trade per year in India (Arunachalam 2011).]

To understand genetic variations in natural populations, it is necessary to examine a large number of polymorphisms. Variations between individuals of species have been observed over millennia and are considered as 'hot-spots' of evolution (Seeni et al. 1998). The study of genetic diversity within species and interspecific or intergeneric relationships among a number of species is carried out with the help of molecular markers (Bandopadhyay et al. 2004). Microsatellites or simple sequence repeats (SSR) occurring in all plant genomes are widely studied for analysing genetic variations in plants. They are abundant in non-coding genomic regions but are also detected in coding regions using SSRs developed from expressed sequence tags (ESTs) (Ranade et al. 2014; Anjali et al. 2015; Sakthipriya and Sabu 2016). Accordingly, SSRs are classified into genomic SSRs and EST-SSRs based on the sequence regions used to identify microsatellite region (Wei et al. 2011).

The genomics of the genus *Calamus* has been reviewed earlier (Arunachalam 2011). Genetic variability in rattans have been reported using random amplified polymorphic DNA (RAPD) (Sreekumar and Renuka 2006; Sarmah et al. 2007), inter simple sequence repeats (ISSR) (Ramesha et al. 2007), and isozyme markers (Ravikanth et al. 2002). There was one attempt to employ microsatellites in rattans (Nageswara Rao et al. 2007), but the study failed to identify significant variations in the samples. The major disadvantage of RAPD and ISSR markers for characterizing population genetics is that heterozygotes cannot be detected (Ferguson et al. 1998). Therefore, the objectives of this study were: (1) to estimate genetic variation in selected *Calamus* species from a wide geographic area in the southern Western Ghats; (2) to analyse the distribution of genetic variability within and among the *Calamus* species; and, (3) to investigate existing patterns of genetic structure in the species.

Materials and methods

Leaf samples from 227 individuals of seven *Calamus* species, namely, *Calamus brandisii* Becc., *C. gamblei* Becc., *C. hookerianus* Becc., *C. metzianus* Schltdl., *C. nagbettai* R.R. Fernald & Dey, *C. thwaitesii* Becc. and *C. travancoricus* Bedd.ex.Becc. were collected from different areas of the states of Kerala and Karnataka (Fig. 1). The field trips were carried out in 2012 − 14 in various natural habitats such as protected areas, buffer zones and periphery adjacent to settlements. Young leaf samples from individual plants were collected throughout their natural habitats at maximum distances but no less than 5 m apart to minimize the chance of collecting clones. Samples were rinsed in running tap water followed by distilled water and 70% ethanol and kept at − 20 °C for DNA extraction.

Microsatellite analysis

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, India). Forty-two microsatellite or simple sequences repeat (SSR) markers were tested, of which 26 were used to assess genetic variability and amplified in all the species (Table 1). The reaction mixture contained 15 pmol each of the forward and reverse primers, 200 µM of deoxynucleotides, 2.5 µL of $MgCl_2$ buffer, 0.2 µL of 5 U/µL Taq polymerase and 50 ng of DNA template. After 2 min at 94 °C, 35 cycles were performed for 30 s at 94 °C, 1 min annealing at the optimized temperature, a 2 min extension at 72 °C, and a final extension of 7 min at 72 °C. The Polymerase chain reaction (PCR) was carried out using SureCycler 8800 (Agilent Technologies, Malaysia).

After the PCR, the amplified DNA products were electrophoresed on 3.5% agarose gel with ethidium bromide (10 µg/µL) and visualized under ultraviolet light using a gel documentation system (UVP Bio-Imaging, UK). To determine the precise PCR product size, a 6% acrylamide gel with silver staining was used (Benbouza et al. 2006). Bands were scored as loci and alleles and used for genetic analysis.

Statistical analysis

The bands obtained with each primer were scored, keeping the expected size range of the PCR products as a reference. The microsatellite data were analyzed using POPGENE version 1.32 (Yeh et al. 1999) and FSTAT version 2.9.3.2 (Goudet 2014). The mean observed number of alleles per locus (*Na*) counts the number of alleles with nonzero frequency. The effective number of alleles per locus (*Ne*) estimates the reciprocal of homozygosity (Hartl and Clark 1989). The Shannon's Information Index (*I*) is a measure of gene diversity (Lewontin 1972). The observed heterozygosity (*Ho*) is a proportion of observed heterozygotes at a given locus and the expected heterozygosity (*He*) is a proportion of expected heterozygotes under random mating (Nei 1973). The percentage of loci that were polymorphic (*P*), regardless of allele frequencies, the observed heterozygosity (*Ho*) and the expected heterozygosity (*He*) were calculated using the POPGENE version 1.32.]

Estimates of *F*-statistics (Weir and Cockerham 1984), average gene diversity within the samples (*Ht*), in all the samples (*Hs*), and among the samples (*Dst*) were obtained

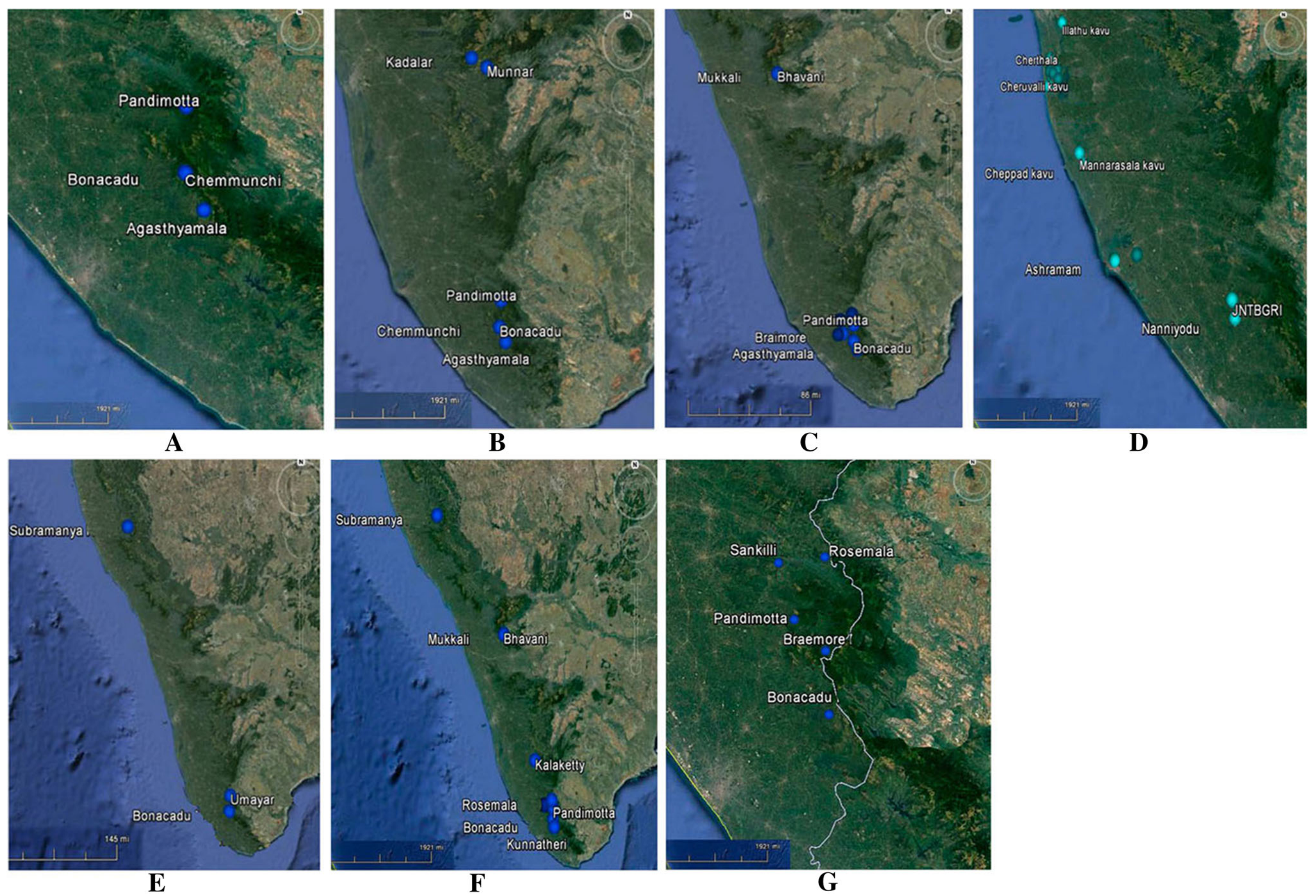

Fig. 1 Map of the sampling location. **a** *Calamus brandisii* (N = 32), **b** *C. gamblei* (N = 30), **c** *C. hookerianus* (N = 36), **d** *C. metzianus* (N = 35), **e** *C. nagbettai* (N = 35), **f** *C. thwaitesii* (N = 42), **g** *C. travancoricus* (N = 17)

using FSTAT software. The unbiased genetic identity and genetic distance were calculated (Nei 1978) as a measure of population structuring. The phylogenetic tree based on Nei (1978) genetic identity estimates was generated in POPGENE version 1.32, following UPGMA (unweighted pair group method with arithmetic average) and visualized using MEGA version 7 (Tamura et al. 2013). STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to analyze population structure, and the 'K' populations were visualized through Structure Harvester (Earl and VonHoldt 2012).

Results

Gene diversity and heterozygosity

Twenty-six SSR primers provided PCR products for the 7 *Calamus* species of a total of 227 accessions. Summary statistics of the loci were tabulated to reveal the informative loci of the variability (Table 2). *Ne* (effective number of alleles per locus) ranged from 1.01 to 2.00 with a mean of 1.29 ± 0.30, *I* (Shannon's Information Index) from 0.02 to 0.69, and the average *Ho* (observed heterozygosity) from 0 to 0.78 with a mean value of 0.16. Average gene diversity *Ht* (within the samples), *Hs* (in all samples) and *Dst* (among the samples) were estimated as 0.13, 0.18 and 0.06 respectively.

Genetic diversity

C. metzianus had the lowest *Ne* value of 1.15; the highest (1.33) was recorded for *C. brandisii*. Estimates of *I* (0.11 and 0.26), *Ho* (0.11 and 0.24), *He* (0.08 and 0.18), and *P* (23.08 and 46.15) exhibited similar ranges for *C. metzianus* and *C. brandisii,* respectively (Table 3).

Genetic structure

The multilocus estimator of *Fst* (theta) (Weir and Cockerham 1984) between all pairs of species (Table 4) was calculated. *C. hookerianus* and *C. travancoricus* showed highest genetic differentiation of 44% as revealed through *Fst* values, whereas the lowest differentiation of 22% was observed between *C. gamblei* and *C. thwaitesii*. The *Fis* estimates were negative (Table 3), indicating low

Table 1 Details of 26 SSR primers, their annealing temperatures and product sizes in seven *Calamus* species

Sl No.	Primer	Primer sequence	Annealing temperature (°C)							Product size (bp)							References
			Cbr	Cgm	Chk	Cmt	Cng	Ctw	Ctr	Cbr	Cgm	Chk	Cmt	Cng	Ctw	Ctr	
1	H19 F	CCTTTGGGACGTCTTTTGAG	52	58	49	50	52	51	50	190	190	190	190	190	190	190	Nadarajah et al. (2009)
	H19 R	CACCCCCATATCGAACATCT															
2	P23 F	CCTTTGGGACGTCTTTTGAG	52	58	49	50	52	52	50	190	190	190	190	190	190	190	
	P23 R	CACCCCCATATCGAACATCT															
3	D09 F	GCCGAAGAGTCCATCAAGAG	49	58	49	50	51	52	50	155	155	155	155	155	155	155	
	D09 R	CTCCATCCACTCCAAATTCC															
4	K07 F	CGAACAGTGCATGGCTTAAA	50	55	49	50	59	50	50	185	185	185	185	185	185	185	
	K07 R	AAAGCACACTTGCACAAACG															
5	006 F	GCATTTGGCAAGAAGAAGGA	50	61	49	50	51	51	50	220	220	220	220	220	220	220	
	006 R	CTGCATGACTCGGTAGTGGA															
6	P09 F	AGAAAGGGAAGTCCCAAAGC	50	58	53	50	50	51	50	170	170	170	170	170	170	170	
	P09 R	TTGCTGCTCTTGCTGAACTC															
7	J09 F	CCTCATGTTACGACGCAGAA	52	61	53	55	52	51	52	150	150	150	150	150	150	150	
	J09 R	AGCACAGGACGGTAATCGAG															
8	K08 F	CGTGAACGGTACGACATTTG	50	58	53	50	51	51	50	200	200	200	200	200	200	200	
	K08 R	CTCACCCACCACTTCTCTCC															
9	P01 F	AGCTTCTTAGCCTCTGTTTCG	50	46	49	50	50	51	49	155	155	155	155	155	155	155	
	P01 R	CTTCTGCTCGGTCTTTTTGG															
10	C3 F	AGAAAGCTGAGAGGGAGATT	52	49	50	50	55	52	50	200	200	200	200	200	200	200	Perera et al. (1999)
	C3 R	GTGGGGCATGAAAAGTAAC															
11	C7 F	ATAGCATATGGTTTTCCT	51	50	49	50	47	51	50	600	280	189	600	440	500	189	
	C7 R	TGCTCCAGCGTTCATCTA															
12	C12 F	ATACCACAGGCTAACAT	52	50	49	50	47	51	50	185	185	185	185	185	185	185	
	C12 R	AACCAGAGACATTTGAA															
13	E2 F	TCGCTGATGAATGCTTGCT	51	49	52	49	50	51	49	177	290	390	200	290	390	290	
	E2 R	GGGGCTGAGGGATAAACC															
14	H4 F	TTAGATCTCCTCCCAAAG	52	46	50	49	45	52	49	300	230	230	230	230	300	230	
	H4 R	ATCGAAAGAACAGTCACG															
15	CHL02 F	CTTCCAAGCTAACGATGC	46	42	54	56	56	49	56	237	237	237	500	237	600	500	Bryan et al. (1999), Cheng et al. (2006)
	CHL02 R	CTGTCCTTAGACAATGTATTCA															
16	CHL05 F	GATGTAGCCAAGTGGATCA	49	51	54	50	50	49	44	163	163	163	163	163	163	163	
	CHL05 R	TAATTTGATTCTTCGTCGC															
17	CHL26 F	GAAAAATGCAAGCACGGTTT	49	54	52	49	46	49	49	124	124	124	124	124	124	124	
	CHL26 R	TACGATCCGTAGTGGGTTCG															

Table 1 continued

Sl No.	Primer	Primer sequence	Annealing temperature (°C)							Product size (bp)							References
			Cbr	Cgm	Chk	Cmt	Cng	Ctw	Ctr	Cbr	Cgm	Chk	Cmt	Cng	Ctw	Ctr	
18	RT11 F	TGGTTAATCGATCGGTCGCC	54	51	46	55	43	56	40	207	207	207	207	207	207	207	Nageswara Rao et al. (2007)
	RT11 R	CGACGGCAGATATACACGC															
19	RT12 F	AGCTTGGGTGATTTCTTGGAAGCG	51	57	53	52	55	50	55	280	280	280	280	157	157	280	
	RT12 R	ACGACGAGGAGTCGCCGTGCAG															
20	RT 13 F	ATGGTGAGAGTTGCTGCCGCCG	49	57	55	55	54	50	55	231	231	231	231	231	231	231	
	RT 13 R	GATGACGCAGAACGGCATCGCC															
21	RT14 F	CCGAACGCCTAGAAGCGCGTCC	45	54	53	55	46	48	52	185	210	185	210	185	185	185	
	RT14 R	CGGCGAGGTTTGCTAATGGCGG															
22	RT15 F	GTCCCCTCCACCCAATTC	51	42	55	55	50	51	40	180	180	180	180	180	180	180	
	RT15 R	TCGTCTACTGTTGGCTGCAC															
23	RT19 F	TCTCCCTCCTCACCATTGTC	52	45	48	52	43	52	50	299	299	299	299	299	299	299	
	RT19 R	TGCTGCCCTCTCTCTCTCTC															
24	RT20 F	CGCATCTCTCTCCCTTATCG	45	50	48	49	42	42	40	178	178	178	178	178	178	178	
	RT20 R	CTCTCCTCCTCGTTGTCGTC															
25	RT21 F	CTGTTGAGCTGGAGAGACCC	50	50	48	49	55	48	49	269	269	269	269	269	269	269	
	RT21 R	CCAACCAGGATCAGTTTGGT															
26	RT24 F	TTACAAGCCACCTCACAAGC	43	45	46	48	41	47	40	337	337	337	337	337	337	337	
	RT24 R	ATACCAGCATCAAGTCAAAAT															

Table 2 Summary statistics of the 26 microsatellite loci used for genotyping the seven *Calamus* species

Locus	*Ne*	*I*	*Ho*	*Hs*	*Ht*	*Dst*	*Dst'*	*Ht'*	*Gst*	*Gst'*	*Gis*
C12	1.01	0.02	0.01	0.01	0.01	0	0	0.01	0.01	0.01	− 0.01
C3	1.68	0.60	0.17	0.21	0.33	0.12	0.13	0.35	0.35	0.39	0.20
C7	1.28	0.37	0.24	0.18	0.27	0.09	0.11	0.28	0.35	0.38	− 0.38
CHL02	1.23	0.33	0.13	0.11	0.19	0.07	0.08	0.20	0.39	0.43	− 0.18
CHL05	1.23	0.34	0.29	0.14	0.25	0.10	0.12	0.26	0.42	0.46	− 1.00
CHL26	1.16	0.27	0.14	0.07	0.13	0.06	0.07	0.14	0.45	0.49	− 0.94
D09	1.29	0.38	0.13	0.18	0.22	0.04	0.05	0.23	0.19	0.22	0.29
E2	1.09	0.18	0.07	0.08	0.09	0.01	0.01	0.09	0.07	0.08	0.22
H19	1.92	0.67	0.59	0.45	0.48	0.03	0.04	0.49	0.07	0.08	− 0.31
H4	1.08	0.16	0.07	0.05	0.06	0.01	0.01	0.07	0.19	0.22	− 0.28
J09	1.01	0.02	0.01	0.01	0.01	0	0	0.01	0.02	0.02	− 0.02
K07	1.06	0.12	0.04	0.06	0.06	0	0	0.06	0.01	0.01	0.26
K08	1.68	0.59	0.08	0.08	0.32	0.25	0.29	0.37	0.77	0.79	− 0.06
O06	1.01	0.02	0	0	0	0	0	0	− 0.01	− 0.01	0.01
P01	1.23	0.34	0.06	0.07	0.14	0.07	0.08	0.16	0.50	0.54	0.15
P09	1.06	0.14	0.03	0.05	0.06	0.01	0.01	0.07	0.19	0.21	0.51
P23	1.70	0.60	0.36	0.32	0.42	0.10	0.12	0.44	0.24	0.27	− 0.13
RT11	1.16	0.26	0.14	0.09	0.13	0.04	0.04	0.14	0.29	0.32	− 0.51
RT12	1.29	0.38	0.16	0.11	0.32	0.20	0.24	0.35	0.64	0.67	− 0.43
RT13	1.72	0.61	0.29	0.18	0.41	0.23	0.27	0.44	0.57	0.61	− 0.63
RT14	2.00	0.69	0.78	0.49	0.50	0.01	0.01	0.50	0.02	0.02	− 0.58
RT15	1.01	0.03	0	0.02	0.02	0	0	0.02	0.03	0.03	1.00
RT19	1.32	0.41	0.15	0.19	0.21	0.02	0.03	0.22	0.10	0.12	0.24
RT20	1.01	0.03	0	0.01	0.01	0	0	0.01	− 0.01	− 0.01	1.00
RT21	1.04	0.09	0	0.02	0.02	0	0	0.02	− 0.01	− 0.01	1.00
RT24	1.14	0.24	0.12	0.08	0.12	0.04	0.05	0.13	0.33	0.36	− 0.47
Mean	1.29	0.30	0.16	0.13	0.18	0.06	0.07	0.19	0.24	0.26	− 0.04
SD	0.30	0.22	0.19	0.13	0.16	0.07	0.08	0.16	0.23	0.24	0.54
Min	1.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	− 0.01	− 0.01	− 1.00
Max	2.00	0.69	0.78	0.49	0.50	0.25	0.29	0.50	0.77	0.79	1.00

Ne, expected number of alleles; *I*, Shannon Information index; *Ho,* observed proportion of heterozygotes; *Hs,* within sample gene diversity; *Ht,* overall gene diversity; and *Dst,* amount of gene diversity among samples. Since this last quantity is dependent on the number of samples, the quantity *Dst*' has been defined independent of the number of samples. *Gst* is an estimator of the parameter *Fst*; *Gst*' is the equivalent estimator, independent of the number of samples; *Gis* is an estimator of *Fis*

inbreeding, i.e., occurrences of outcrossing were more prevalent than inbreeding).

The mean genetic identity among populations was 0.92 (SD = 0.024; Table 5). The highest genetic identity was between *C. metzianus* and *C. travancoricus* (0.95) and the lowest between *C. brandisii* and *C. nagbettai* (0.88). The dendrogram using Nei's genetic distance (Nei 1978) showed two groups: one with *C. hookerianus,* the other with the remaining 6 species (Fig. 2). The observed clustering does not have a relationship with the habit and systematic position. However, the dendrogram created using Nei's genetic identity data from the 227 accessions showed them generally into the respective biological species (Fig. 3).

STRUCTURE HARVESTER software was used to analyse the population structure in the 227 individuals. The program applies a Bayesian model-based clustering algorithm that identifies subgroups with distinct allele frequencies. This procedure places individuals into K clusters, where K is chosen in advance but may be varied across independent runs of the algorithm. The program visualized the results of the analysis and determined the 'K' clusters. Individuals were assigned to populations or multiple populations if their genotype indicated an admixture. The highest-likelihood run for K 7 found distinct structure where the seven species exactly clustered into seven groups with some differentiation or sub-structuring (Fig. 4).

Table 3 Species-wise descriptive statistics and extent of inbreeding as evidenced from *Fis* values obtained using 26 SSR markers in seven *Calamus* species

Locus	*Ne*	*I*	*Ho*	*He*	*P*	*Fis*
C. brandisii	1.33	0.26	0.24	0.18	46.15	− 0.331
C. gamblei	1.17	0.16	0.11	0.10	38.46	− 0.015
C. hookerianus	1.20	0.17	0.14	0.12	42.31	− 0.243
C. metzianus	1.15	0.11	0.11	0.08	23.08	− 0.363
C. nagbettai	1.28	0.22	0.19	0.15	38.46	− 0.276
C. thwaitesii	1.20	0.18	0.13	0.12	38.46	− 0.094
C. travancoricus	1.22	0.19	0.16	0.13	42.31	− 0.303

Ne, effective number of alleles per locus; *I*, Shannon's Information Index; *Ho*, the observed heterozygosity; *He*, expected heterozygosity; *P*, percentage of all polymorphic loci; *Fis*, inbreeding coefficient

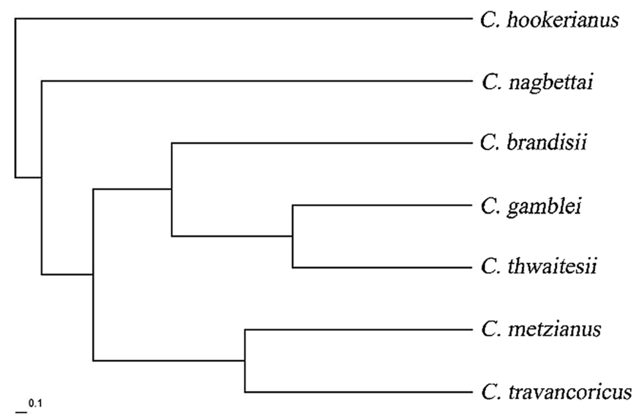

Fig. 2 Dendrogram based on Nei's genetic distance (Nei 1978) using 26 SSR markers in seven *Calamus* species. Method = UPGMA. Modified from NEIGHBOR procedure of PHYLIP Version 3.5

Discussion

Amount of genetic variation

Allelic variation may be detected and estimated using genetic markers; however, the value of genetic markers for detecting population differences depends largely on the differences of the alleles in various individuals of a population (Nei 1987). Different methods have been adopted in the last two decades to reveal the extent of genetic diversity in various *Calamus* species (Bon 1996; Changtragoon et al. 1996; Ravikanth et al. 1999; Wickneswari et al. 2002; Sudarmonowati et al. 2004; Sarmah 2005; Sreekumar and Renuka 2006; Nageswara Rao et al. 2007; Sarmah et al. 2007; Ramesha et al. 2007; Ambida et al. 2012; Swain and Abhijita 2015; Priya et al. 2016). In a pioneering study carried out in Malaysia during the mid-1990s, allozymes from *C. subinermis* were characterized and polymorphism was much higher (70.5%) in this species than expected levels of genetic diversity for tropical woody and non-woody species (Bon 1996). RAPD markers have also been employed during this period to study genetic variability of

Table 4 Genetic differentiation (*Fst*) between pairs of *Calamus* species

	C. brandisii	*C. gamblei*	*C. hookerianus*	*C. metzianus*	*C. nagbettai*	*C. thwaitesii*	*C. travancoricus*
C. brandisii	0	0.2303	0.3133	0.3546	0.3627	0.2895	0.2697
C. gamblei	0.2303	0	0.3987	0.3328	0.2814	0.2168	0.3848
C. hookerianus	0.3133	0.3987	0	0.3733	0.4109	0.4336	0.4447
C. metzianus	0.3546	0.3328	0.3733	0	0.3602	0.3682	0.3065
C. nagbettai	0.3627	0.2814	0.4109	0.3602	0	0.2986	0.3955
C. thwaitesii	0.2895	0.2168	0.4336	0.3682	0.2986	0	0.4102
C. travancoricus	0.2697	0.3848	0.4447	0.3065	0.3955	0.4102	0

Table 5 Genetic identity (above **** diagonal) and genetic distance (below **** diagonal) estimated using 26 SSR markers in seven *Calamus* species

	C. brandisii	*C. gamblei*	*C. hookerianus*	*C. metzianus*	*C. nagbettai*	*C. thwaitesii*	*C. travancoricus*
C. brandisii	****	0.9477	0.9204	0.9175	0.8816	0.9279	0.9235
C. gamblei	0.0537	****	0.9178	0.9489	0.9399	0.9626	0.9174
C. hookerianus	0.0829	0.0858	****	0.9350	0.8912	0.8941	0.8883
C. metzianus	0.0861	0.0525	0.0672	****	0.9245	0.9311	0.9523
C. nagbettai	0.1260	0.0620	0.1152	0.0785	****	0.9328	0.8847
C. thwaitesii	0.0748	0.0381	0.1119	0.0714	0.0696	****	0.8983
C. travancoricus	0.0796	0.0862	0.1184	0.0489	0.1225	0.1073	****

Fig. 3 Nei's genetic identity-based dendrogram using 26 SSR markers in 227 accessions belonging to seven *Calamus* species. Cbr: *C. brandisii,* Cgm: *C. gamblei,* Chk: *C. hookerianus,* Cmt: *C. metzianus,* Cng: *C. nagbettai,* Cth: *C. thwaitesii,* Ctr: *C. travancoricus*

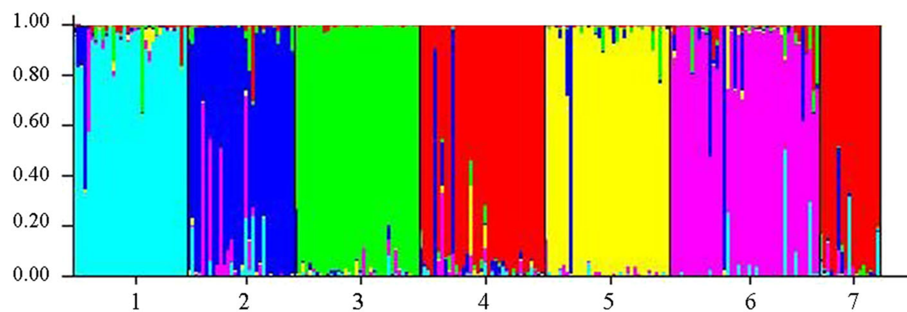

Fig. 4 Model-based population structure of 227 individuals of *Calamus* spp. Numbers indicate the populations formed based on the genetic characteristics which are the same as that of the biological species. 1: *C. brandisii,* 2: *C. gamblei,* 3: *C. hookerianus,* 4: *C. metzianus,* 5: *C. nagbettai,* 6: *C. thwaitesii,* 7: *C. travancoricus*

Calamus species of Thailand (Changtragoon et al. 1996). Isozyme analysis in *C. manan* from Malaysia showed extremely low polymorphism (6.7 to 20.0%) for mature plants from rattan plantations (Wickneswari et al. 2002). On the other hand, natural populations of this species in Indonesia exhibited higher isozyme diversity with respect to polymorphic loci, ranging from 66.7 to 76.7% (Sudarmonowati et al. 2004). In a study of north-eastern Indian *Calamus* species viz. *C. flagellum*, *C. gracilis*, *C. khasianus*, *C. tenuis*, *C. erectus*, *C. leptospadix*, *C. inermis*, and *C. acanthospathus*, average polymorphism of 93.0 and 98.1% were identified using isozymes and RAPDs, respectively (Sarmah 2005), which indicated a better scope for genetic improvement of rattans in this region. Allozyme analysis involving 12 loci over eight enzyme systems detected polymorphic loci in *C. thwaitesii* from the central Western Ghats in India's Karnataka state ranging from 75.0 to 91.7% (Ravikanth et al. 1999). In another study, the percentage of polymorphic loci varied from 40.0 to 60.8% in eight populations of *C. thwaitesii* from the Western Ghats and Sri Lanka using RAPD markers (Sreekumar and Renuka 2006). The genetic distance between these populations ranged from 0.03 to 0.3. However, ISSR (inter simple sequence repeats) analysis of *C. thwaitesii* in the central Western Ghats showed an average of 45.3 polymorphism. Greater diversity was found in the core protected areas in comparison to the buffer and periphery regions (Ramesha et al. 2007). Molecular analyses of different Indian and Sri Lankan populations of *C. rivalis* and *C. metzianus* carried out using RAPD markers revealed that the percentage of polymorphic loci between populations varied from 26.5 to 68.4%, and the genetic distance between populations ranged from 0.1 to 0.3 (Sreekumar et al. 2006). This study helped to resolve the taxonomic ambiguity previously existing in these two species and recommended that *C. rivalis* be merged with *C. metzianus*. Preliminary molecular analysis of twelve species of *Calamus* from The Philippines using modified RAPD and ISSR primers indicated 100% polymorphism in all the loci tested (Ambida et al. 2012). Another study of six *Calamus* species from Orissa, India using ISSR primers, also exhibited high (94.6%) polymorphism (Swain and Abhijita 2015). Recently, ISSR analysis of *C. vattayila*, a medium sized rattan endemic to the Western Ghats, showed an average polymorphism of 36.2% in three populations studied (Priya et al. 2016).

In the above studies, either isozymes, RAPD or ISSR markers were used. For the first time, in an attempt to identify microsatellite markers to determine gene flow events and genetic differentiation of populations in rattans, those developed for *Cocos nucifera* L. (family Arecaceae) were examined for cross-species amplification (Nageswara Rao et al. 2007). Of the six microsatellite primer pairs screened, two yielded good cross-species amplification across different rattan genera and species from South and Southeast Asia. In this study, low levels of genetic variability were recorded which was not surprising for EST-SSR markers. The proportion of polymorphic loci ranged from 23.1 to 46.2 for *C. metzianus* and *C. brandisii* respectively. The surprisingly low polymorphism shown by *C. metzianus* might be that the samples were collected mostly from sacred groves (regions of micro forest, rich in biodiversity and protected by local communities), and the rattan population in these areas are represented by a few individuals, limiting continued evolution. These plants might be introduced to develop different sacred groves in the past from the same habitat. Average polymorphism for the remaining six species was 41.0%.

Distribution of genetic variability

Generally, outcrossing species retain most of their genetic variability within populations (Bartish et al. 1999). This is true for *Calamus* species as reported from several studies. The levels of population differentiation in *C. thwaitesii* are low, as indicated by the mean *Fst* value of 8.8% generated through allozyme analysis, suggesting that nearly 91.0% of the genetic variation is accounted by variation within a population (Ravikanth et al. 2001). In another study using RAPD markers, the majority of the genetic diversity was distributed within populations (70.8%) and only (29.2%) among populations of *C. thwaitesii* (Sreekumar and Renuka 2006). An analysis of molecular variance (AMOVA) of variations within and between populations of *C. rivalis* and *C. metzianus* of populations of the Western Ghats treated as one region and the Sri Lankan populations as another, showed that the percentages of variation attributable to the differences between regions, among populations within regions, and among individuals within populations were 18.8% ($p < 0.001$), 21.4% ($p < 0.001$), and 59.9% ($p < 0.009$), respectively, (Sreekumar et al. 2006). This study revealed a coefficient of gene differentiation (*Gst*) measures the gene diversity between populations of 24.0%, indicating that 76% of variation resides within populations (Table 2).

Distribution of genetic variability over space is critical in conservation. Generally moderate to high genetic diversity in the core areas results in higher fitness of small populations and is noted in this study in species such as *C. brandisii* ($p = 46.2\%$; Table 3) which was collected from the Agasthyamalai Biosphere Reserve. This was also observed in a study of *C. thwaitesii,* where populations sampled from core and buffer regions maintained better population structure as well as higher genetic diversity than populations of the peripheral regions of the protected area (Ramesha et al. 2007).

Genetic structure

Sampling from predefined populations is used mostly for studies of human genetic variation where populations are usually defined on the basis of culture or geography and might not reflect underlying genetic relationships (Foster and Sharp 2002). On the other hand, sampling of plants to analyse genetic structure mostly considers geographical boundaries of the distribution. Analysis of multilocus genotypes allows inference of genetic ancestry without relying on information on sampling locations of individuals (Bowcock et al. 1994; Mountain and Cavalli-Sforza 1997; Pritchard et al. 2000). The 227 individuals of the *Calamus* species collected from various locations in this study were analysed using STRUCTURE software without considering their biological status. Surprisingly, the seven species separated into seven K groups (Fig. 4). However, separation shows different levels of sub-structuring within each species. The UPGMA-based dendrogram using genetic identity estimates also resulted in a similar profile with varying levels of differentiation (Fig. 3). Generally, the breeding system determines the extent of within-population genetic diversity, whereas other factors, including geographical distribution, have little effect (Bartish et al. 1999). However, this concept does not account for genetic drift due to over exploitation of the mature *Calamus* species that usually occurs from the periphery of the protected areas. In the long-term, this would affect the continued evolution of the species but an outcrossing mechanism would reconcile some of the loss and help to restore genetic variability to some extent for these dioecious species.

Protected areas, genetic diversity hotspots and conservation implications

The amount of genetic variation within a species in its natural habitat determines the niches or hotspots. The Western Ghats, a 1600 km mountain chain between 8 to 21°N parallel to the west coast of India and older than the Himalayas, is considered one of the eight 'hottest hotspots' of biological diversity in the world (Daniels 1992; Myers et al. 2000), and is listed in the UNESCO World Natural Heritage sites July 2012. Identifying lesser areas of high genetic diversity and endemism, or micro-hotspots within larger hotspots at diverse scales, has been carried out for both flora and fauna (Murray-Smith et al. 2006; Raes et al. 2009; Kraft et al. 2010; Schouten et al. 2010; Lopez-Lopez et al. 2011).

Except for *C. metzianus*, all the species were collected from various regions of the Western Ghats (Table 6). An attempt was made to identify micro-hotspots for these species. *C. brandisii* showed the highest heterozygosity (*Ho*) of 0.40 from the Agasthyamala Biosphere Reserve. High and low altitude provenances of *C. brandisii* determine the clear ecotypic differentiation among the species. Even though *C. gamblei* is more widely distributed than *C. brandisii* due to its occurrence in a narrow elevation range, the species displayed an observed heterozygosity of 0.24 from the Bonacaud region of the Reserve. The occurrence of *C. hookerianus* from the buffer areas to the periphery maintained an observed heterozygosity of 0.23 from the region. The higher occurrence of *C. travancoricus* produced a higher heterozygosity of 0.28 compared to *C. gamblei* and *C. hookerianus* from the Rosemala area of the Shenduruney Wildlife Sanctuary. Due to the broad pattern of distribution of C. *thwaitesii* from the southern and central Western Ghats, the species had a significantly higher heterozygosity; the highest of 0.32 was recorded from the Rosemala area. The universally threatened, endemic species, *C. nagbettai,* showed a heterozygosity of 0.29 from the Subramanya forest of the Pushpagiri Wildlife Sanctuary. The protected areas in the Agasthyamala Biosphere Reserve have higher species density, greater gene flow and higher regeneration due to fewer human interventions and to the intrinsic spatial distribution gradients of the species. Detailed location-wise heterozygocity values are given in Table 6.

The genetic diversity hotspots of rattans in the southern and central Western Ghats were found in the micro-hotspots of the Agasthyamala Biosphere Reserve and the Pushpagiri Wildlife Sanctuary (Table 7). The intra-specific genetic variation was found below the Achenkovil gap in the Agasthyamala, Bonacaud and Rosemala regions and above the Palghat gap in the Subramanya forest. In the Western Ghats, protected areas are well-managed and conserved. However, they are surrounded by large numbers of villages and the boundaries are permeable, leading to the illegal harvesting of rattans. Three of the four micro-hotspots were identified in the southern part of the Western Ghats and one from the central Western Ghats. The four hotspots are at low, mid-to high elevations in the southern Western Ghats, and low-to mid-elevations in the central Western Ghats. These protected areas may be possible for future reintroduction and species recovery programs.

Conclusions

Varying levels of genetic diversity were found for different *Calamus* species depending on their areas of distribution, their exploitation for commercial use, and level of protection status. The findings of this study provide a strong foundation for further strengthening protected area networks as well as the enrichment of man-made micro habitats such as sacred groves, especially areas of intermediate levels of disturbances. The dioecious nature of

Table 6 Observed heterozygosity in seven *Calamus* species using 26 microsatellite markers

Sampling locations	*Calamus* species						
	Calamus brandisii	*Calamus gamblei*	*Calamus hookerianus*	*Calamus metzianus*	*Calamus nagbettai*	*Calamus thwaitesii*	*Calamus travancoricus*
Agasthyamala	0.400	0.136	0.231			0.217	
Bonacaud	0.292	0.238	0.200		0.105	0.125	0.211
Pandimotta	0.269	0.115	0.167			0.167	0.227
Rosemala	0.240		0.167			0.320	0.280
Brimore			0.167				0.182
Chemmunji		0.083					
Kalaketty						0.192	
Mukkali			0.120			0.269	
Kadalar		0.174					
Munnar		0.167					
Cherthala				0.182			
Ashramam				0.167			
Nanniyode				0.130			
Nallilakavu				0.048			
Subramanya					0.292	0.154	
Umayaar					0.174		

Table 7 Seven *Calamus* species of the Western Ghats with conservation status and region of genetic diversity micro-hotspots

SL	Species	Endemic/ Non-endemic	Elevation (m)	Gps coordinates	Micro-hot-spot	Protected site	Regions
1	*C. brandisii*	Endemic	1000–2000	N 08°37″05.01″ E 077°13″37.52″	Agasthyamala	Agasthyamala Biosphere reserve	S
2	*C. gamblei*	Endemic	500–1000	N 08°41″33.73″ E 077°10″50.35″	Bonacaud	Agasthyamala Biosphere reserve	S, C
3	*C. hookerianus*	Endemic	Up to 1000	N 08°35″28.49″ E 077°11″09.67″	Agasthyamala	Agasthyamala Biosphere reserve	S, C
4	*C. metzianus*	Endemic	50–100	N 09°40″34.16″ E 076°18″35.68″	NA	Sacred groves	S, C
5	*C. nagbettai*	Endemic	Up to 1000	N 12°40″17.8″ E 075°33″35.4″	Subramanya	Pushpagiri wildlife santuary	S, C
6	*C. thwaitesii*	WG/SL	100–900	N 08°55″10.57″ E 077°10″30.80″	Rosemala	Shenduruney wildlife santuary	S, C, N
7	*C. travancoricus*	Endemic	300–1000	N 08°54″49.6″ E 077°10″55.2″	Rosemala	Shenduruney wildlife santuary	S, C

WG Western Ghats, *SL* Srilanka, *S* Southern Western Ghats, *C* Central Western Ghats, *N* Northern Western Ghats, *NA* not analysed for genetic diversity micro-hotspots

Calamus species may reconcile some of the genetic loss and help to restore genetic variability over a period of time. The present study provides useful information for genetic conservation and will aid in further understanding of species phylogeography on the Indian subcontinent.

Acknowledgements We extend our sincere thanks to the Director, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), for providing necessary facilities and the Kerala State Forest Department for granting permission to collect rattan leaf samples from various forest regions of Kerala (Order No. WL 10-3053/2012).

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