

1 **Analysis of genetic diversity among the five species** 2 **of *Calamus* by RAPD markers.**

3 **ABSTRACT:**

4 **Aims:** the aim of the present study was to determine genetic diversity among the five commercially
5 important species of *Calamus* using RAPD markers.

6 **Study Design:** DNA extracted from mature leaves of selected species was used as template DNA for
7 optimization of PCR amplification. Data was analysed with the help of software-numerical taxonomy
8 and multivariate analysis version 2.02e (NTSYSpc) and unweighted pair group method with
9 arithmetic mean (UPGMA).

10 **Place and duration of study:** The study was carried out at Department of Botany, Bangalore
11 University, Bangalore 560056, India during 2015.

12 **Methodology:** *Calamus huegelianus*, *C. nagabettai*, *C. prasinus*, *C. thwaitesii* and *C. vattayila*,
13 known for their good quality canes, are selected for the present study to characterize them through
14 RAPD analysis. Fifteen decamer primers were used for amplification. However, only five primers
15 have generated clear reproducible bands. Simple matching, similarity co-efficient, dendrogram and
16 principal component analysis were composed by using NTSYSpc and UPGMA.

17 **Results:** Clear and reproducible 108 bands were scored with 64 polymorphic bands indicating 59.25
18 per cent of polymorphism among the species selected. Higher per cent of 76.00 was noted in
19 *C. prasinus* and least of 43.67 in *C. huegelianus*. Similarity co-efficient value of 0.45 to 0.66 indicates
20 the less genetic divergence among the taxa selected. The cluster analysis with the help of dendrogram
21 and principal component analysis has revealed that *C. vattayila* is distinct from others since it has 6
22 veined leaflets. The other selected five taxa are having three veined leaflets. Hence clustering pattern
23 was compared with distinguished phenotypic characters and dichotomous key is proposed for the taxa
24 studied.

25 **Conclusion:** The previously mentioned data obtained through cluster and principal component
26 analysis clearly indicate that the taxa are clustered together based on sharing of common phenotypic
27 characters. The present study is a step forward in understanding the genetic affinities among the
28 selected taxa and their conservation strategies.

29 **Keywords:** *Calamus*; Dichotomous key; Polymorphism; RAPD; Rattans.

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32 1. INTRODUCTION

33 *Calamus* is one of the important non-timber products (NTFP) of tropical forests of India
 34 categorised under Rattans along with other three genera - *Daemonorops*, *Korthalsia* and
 35 *Plectocomia* of the family Arecaceae. For years, rattans have played a major role in providing
 36 job opportunities and enhancing the economic situation of both tribal and other forest
 37 dwelling communities. It is estimated that more than a half a million people are directly
 38 employed in harvesting and processing of rattans in the rural areas of South-East Asian
 39 countries [1, 2]. Most of the raw material for local processing and for supplying the rattan
 40 industry is still obtained by harvesting of unmanaged, wild rattan resources in natural tropical
 41 forests. Hence, both government and non-government organizations formulate several action
 42 programmes and strategies for the base line information on conservation, sustainable
 43 management of genetic resources and utilization. Seed is the best source of propagation in
 44 these taxa but its availability on a large scale is dependent on certain factors. Extraction of the
 45 plants before flowering and destruction of the natural habitats have led to the depletion of the
 46 germplasms of these taxa.

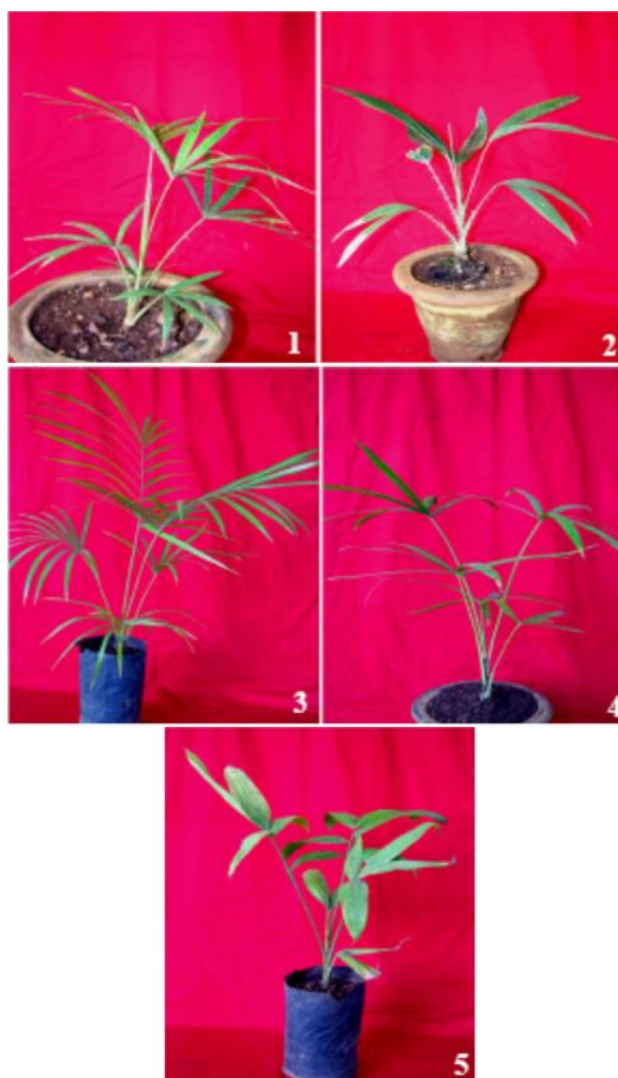
47 *Calamus* L. popularly known as cane is represented by 370 species distributed in three major
 48 geographical regions in India – Western Ghats of peninsular region, Sub-Himalayan hills and
 49 Andaman and Nicobar islands [3]. Species of *Calamus* are wind pollinated with their
 50 phenological behaviour being influenced by climatic, topographical, and edaphic factors.
 51 *Calamus* is dioecious and flowers only once in a year. The low frequency of male plants and
 52 wastage of pollen during rains that led to decreased pollination efficiency and low seed set [4,
 53 5].

54 *Calamus huegelianus*, *C. nagabettai*, *C. prasinus*, *C. thwaitesii* and *C. vattayila* are the taxa
 55 selected for the present study (Fig. 1). *C. huegelianus* is endangered, while *C. nagabettai* and
 56 *C. vattayila* are endemic to Western Ghats. *C. prasinus* and *C. thwaitesii* are comparatively
 57 having wide distribution. However, all the five species of *Calamus* selected for the present
 58 study are economically important and are extensively used in furniture industry that led to
 59 their threatened status. *C. huegelianus*, *C. nagabettai* and *C. thwaitesii* are clump-forming
 60 canes, whereas *C. prasinus* and *C. vattayila* are solitary canes. Morphological analysis of all
 61 these five taxa selected revealed the existence of distinct variations both within and among
 62 the taxa. Taxonomically *Calamus* species are heterogeneous plants displaying considerable
 63 morphological variability [6, 7, 8]. The assessment of genetic diversity of *Calamus* in
 64 Western Ghats is a prerequisite to overcome threat to the genetic diversity present in wild

65 populations and need for preservation of their genetic resources. Further, genetic diversity
66 studies in biodiversity hotspots like Western Ghats, aids in identifying gene erosion, priority
67 locations, and species for conservation [9].

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72 Fig-1

73 Randomly amplified polymorphic DNA (RAPD) has been extensively used for the
74 assessment of genetic diversity in a variety of plants including *Calamus* species [2, 10-14].
75 RAPD analysis is technically simple, less expensive, and quick to perform, requires very little
76 plant material, and yields true genetic markers [15]. Further previous knowledge of the gene

sequence of the sample is not required to perform the analysis. RAPD analysis provides information that can help to define the distinctiveness of species and phylogenetic relationships at the molecular level [13]. Reports on genetic analysis in *Calamus* species are limited [2, 13, 16-19].

The present investigation is an attempt to study the genetic relatedness/diversity among *C. huegelianus*, *C. nagabettai*, *C. prasinus*, *C. thwaitesii* and *C. vattayila* and to correlate the phenotypic characters with the data obtained from genetic analysis by RAPD markers.

2. MATERIAL AND METHODS

2.1 Sample collection for DNA isolation

Seedlings were collected from Subramanya range of Western Ghats and maintained in the polyhouse of the departmental garden(Fig.1). Mature leaves from one-year-old seedlings of all the selected species of *Calamus* were used for DNA extraction. Total DNA was extracted from 500mg of fresh leaves using the modified Laird method [20]. Further, the DNA sample was column purified to remove the inhibitors for PCR.

2.2 PCR-RAPD

The genomic DNA of five samples was used as template DNA for optimization of PCR amplification. Amplification was achieved by following the procedure outlined by Williams et al. [21] with slight modification. 2µl of different template DNA was added to the 38 µl reaction mixtures which contained 2 x PCR master mixes of 20 µl, 1µl of random primer, and 17µl of deionised distilled water. PCR master mix is composed of 1.5µl of MgCl₂, 1.00µl of dNTPS, one unit of taq DNA polymerase. The master mix required for a set of 10 reactions was prepared fresh from the original stock. 40µl of this mixture was aliquoted into 48 different labelled PCR vials. Amplification was achieved by Ependorf master thermocycler programmed for initial denaturation at 94° C for 5 min followed by 40 cycles each: each cycle consisting of denaturation at 94°C for 30s, primer annealing at 45°C for 60s, primer extension at 72°C for 90s and a final extension for 7 min at 72°C. Five random primers were selected – OPB-10, OPD-02, OPD-08, OPC-06, and OPC-07 out of 15 primers tried for finger printing. They yielded maximum number of bands that are consistent and clear without any ambiguity.

Amplification products were resolved by electrophoresis in 0.8% agarose gel containing ethidium bromide. Ethidium bromide stained DNA bands were viewed under UV transilluminator – Syngen G bio gel documentation system and photographed for documentation.

2.3 Data analysis

The amplification products were scored for the presence (1) and absence (0) of bands across the species to generate binary matrix. Per cent polymorphism was calculated by the following formula,

$$\text{Per cent polymorphism} = \frac{\text{Number polymorphic bands}}{\text{Total number of bands}} \times 100$$

The dendrogram and Principal component analysis (PCA) were generated by calculating similarity matrix (simple matching) and corresponding clustering by UPGMA using Numerical taxonomy and multivariate analysis version 2.02e software (NTSYSpc) [22].

3. RESULTS

In the present study, five primers were used to assess the morphological variation at the molecular level (Fig. 2). The size range of amplification products differed with selected primer as well as genotypes, which ranged from 100bp – 3.5kb. The RAPD bands produced by individual primer with different samples are tabulated (Table 1). Five primers used in the present study have generated 108 bands. 64 bands were found to be polymorphic which represents 59.25% of polymorphism. The average number of bands and polymorphic markers per primer were found to be 21.6 and 12.8 respectively. *C.prasinus* has exhibited highest per cent polymorphism with 11 polymorphic bands out of 16. Least per cent of polymorphism was recorded in *C.huegelianus* with 9 polymorphic bands out of 20. Among the five primers, OPD-02 produced least per cent polymorphism of 30.76 with least number of 13 bands. The highest per cent of polymorphism of 82.60 was generated by OPC-07 (Table 2). The molecular weight of RAPD markers ranges from 1123 to 614bp in the profile generated by primer OPD-02. However, OPD-08 has generated 29 markers whose molecular weight ranges from 1322 to 342bp. Maximum number of 30 RAPD markers was generated by primer OPC-06 with 63.33 % polymorphism, whose molecular weight ranges from 2564 to 329bp, while the primer OPC-07 generated 23 markers with molecular weight ranging from 1310 to 388bp. The primer OPB-10 generated least number of 13 bands with molecular weight ranging from 1432 to 615bp.

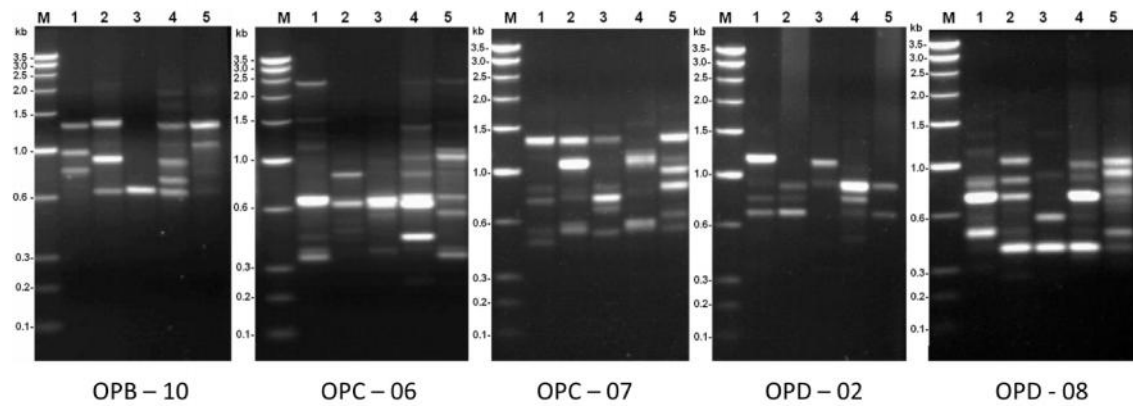


Fig 2 : RAPD Profiles generated by 5 Primers

M Marker 1. *C. nagabettai*, 2. *C. huegelianus*, 3. *C. prasinus*, 4. *C. thwaitesii*, 5. *C. vattayila*

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Table 1: Total number of RAPD and Polymorphic markers in RAPD profile

Primers	Number of RAPD bands					Number of Polymorphic bands				
	<i>C. nagabettai</i>	<i>C. huegelianus</i>	<i>C. prasinus</i>	<i>C. thwaitesii</i>	<i>C. vattayila</i>	<i>C. nagabettai</i>	<i>C. huegelianus</i>	<i>C. prasinus</i>	<i>C. thwaitesii</i>	<i>C. vattayila</i>
OPD-02	3	3	2	3	2	1	0	2	0	1
OPD-08	6	6	4	6	7	4	2	2	2	4
OPC-06	7	2	4	8	9	4	1	2	5	7
OPC-07	5	5	5	2	6	4	3	4	2	6
OPB-10	3	4	1	3	2	2	3	1	1	1
Total bands	24	20	16	22	26	15	9	11	10	19

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Table 2: Summary of RAPD analysis in five species of *Calamus*

Sl No.	Primers	Per cent Polymorphism					Total No. of RAPD bands	Total No. of Polymorphic bands	Per cent polymorphism
		<i>C. nagabettai</i>	<i>C. huegelianus</i>	<i>C. prasinus</i>	<i>C. thwaitesii</i>	<i>C. vattayila</i>			
1	OPD-02	33.33	00.00	100.00	00.00	50.00	13	04	30.76
2	OPD-08	66.66	33.33	50.00	33.33	57.14	29	14	48.27
3	OPC-06	57.14	50.00	50.00	62.50	77.77	30	19	63.33
4	OPC-07	80.00	60.00	80.00	100.00	100.00	23	19	82.60
5	OPB-10	66.66	75.00	100.00	33.33	50.00	13	08	61.15
Total		60.76	43.67	76.00	45.83	66.98	108	64	59.25

3.1 Cluster and Principal Component Analysis

The RAPD bands were manually scored from the gel profile by assigning values '1' for the presence and '0' for the absence and binary matrix was generated. Thus, generated binary matrix from all five profiles generated by five primers was used for statistical analysis. Simple matching, similarity co-efficient and dendrogram were computed by using a software numerical taxonomy and multivariate analysis version 2.02 e and unweighted pair group method with arithmetic mean (UPGMA). The similarity co-efficient value in the distance matrix ranged from 0.45 to 0.66 indicating less genetic divergence among the taxa selected. Highest similarity co-efficient of 0.66 is shown between *C.huegelianus* and *C.thwaitesii*, whereas the least similarity co-efficient of 0.45 is between *C.vattayila* and *C.nagabettai*. The dendrogram generated by overlapping the data obtained from five primers, generated two main clusters 'a' and 'b' (Fig. 3). Clusters 'a' is represented by *C.vattayila* that is stood out from the rest of the species selected. Cluster 'b' is again divided into 'c' and 'd'; cluster 'd' is represented by *C.nagabettai* alone. Cluster 'c' is further split into 'e' and 'f' sub clusters; "e" is composed of *C.prasinus* and 'f' cluster is composed of *C.huegelianus* and *C.thwaitesii*. PCA result corresponds with the grouping of taxa based on cluster analysis. *C.huegelianus* and *C.thwaitesii* are closely related, whereas *C.nagabettai* and *C. vattayila* have fallen apart in the 3-D plot (Fig.4).

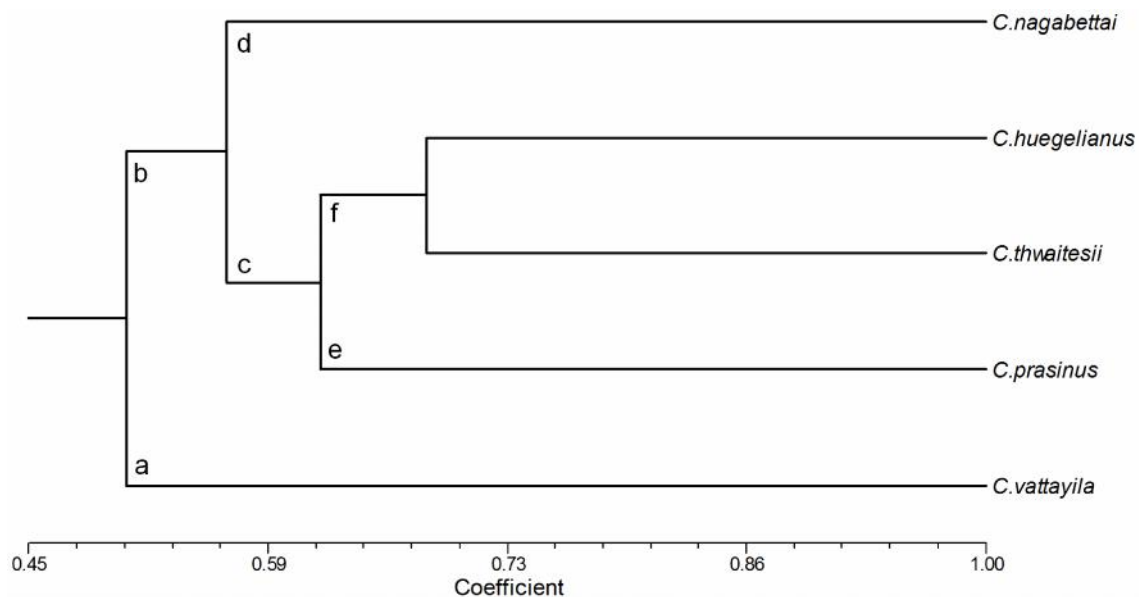


Fig-3

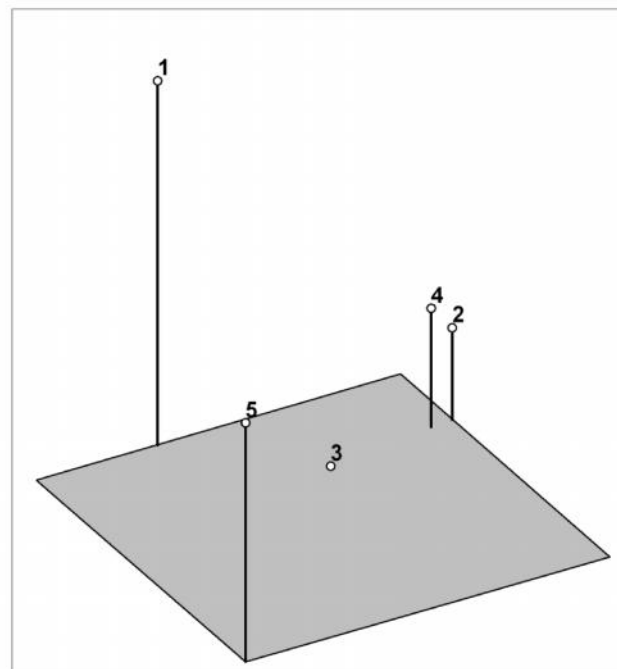


Fig-4

4. DISCUSSION

The depletion of genetic resources from natural habitats due to various anthropogenic activities in Southeast Asian countries has led to a serious threat to the rattan trade including India [23]. Hence, there is a need to conserve genetic resources of these species. *In situ* and *ex situ* conservation strategies are depended on the knowledge of the genetic structure, diversity of populations and mating system of the species. Information obtained from genetic marker screening is invaluable for identification of populations, which are desirable for forestry practices that inadvertently alter natural gene pool of domesticated species [17]. Genetic richness can be assessed by estimating the genetic diversity parameters like percent of polymeric loci and genetic diversity index.

Various PCR-based marker techniques have been successfully introduced in the fingerprinting of plant genome [24] and in genetic diversity studies [25]. Among them Random amplified polymorphic DNA analysis (RAPD) is quick and well adopted for efficient non-radioactive DNA finger printing of genotypes [26]. RAPD has advantage over

other markers, as the knowledge of DNA sequence for the targeted genome is not required. This makes the method more popular for comparing the DNA of biological systems that have not had the attention of the scientific community or in a system in which relatively few DNA sequences are compared. In addition to this RAPD is inexpensive yet powerful method compared to other marker system in analysing phylogeny of diverse plant and animal species.

The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationships in several genera [10, 27]. The RAPD markers are well suited for genetic mapping, for plant and animal breeding applications and for DNA fingerprinting with particular utility for studies of population genetics [21]. Williams et al. [21] who for the first time described these markers have listed several advantages of this technique. Significant among them are as follows : 1) a universal set of primer can be used for genomic analysis in a wide variety of species, 2) no preliminary work, such as isolation of cloned DNA probes, preparation of fitters for hybridization or nucleotide sequence is required 3) each RAPD marker is the equivalent of sequence tagged site 4) which can greatly simplify information, transfer in collaboration research programme.

Five different species of *Calamus* were selected for the present study. The selected taxa are all economically important and are over exploited. Rattans are heterogeneous group of plants displaying considerable morphological variability [6, 7, 8]. Morphological analysis of the five taxa selected revealed the existence of variations both within and among the taxa [28].

Mature fresh leaves of all the five taxa were used for the DNA extraction. Stored samples were found to yield DNAs of reduced quality and quantity, which may be due to the formation of several irreversible complexes between polyphenols and DNA [29]. The isolation of genomic DNA and its purity play an important role in further processing. Removal of RNA and proteins is an important step in DNA purification, since they may hinder PCR amplification. The isolate DNA was column purified to remove any inhibitors that may come in the way of PCR reaction.

DNA amplification was tried with different concentrations of genomic DNA. Out of these 2 µl of template DNA gave good and constant amplification. 1 µl of primer was found optimal. Higher primer concentration may promote mispriming and accumulation of non-septic products [30]. Both the magnesium ion and dNTPs concentrations are known to affect the relative intensity and the number of amplified bands. It was found that 1.5 µl of Mgcl₂

and 1.00 µl of dNTPs was optimal. At higher Mg ion concentration, Taq polymerase activity is inhibited [31].

Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual plants. For the present study five operon primers were selected and they produced 108 bands. Similarity matrix data has shown that *C. thwaitesii* and *C. nagabettai* share 58% similarity whereas *C. vattayila* and *C. prasinus* with 50% similarity have branched out separately. Both these species are solitary canes.

Analysis of RAPD profile revealed that a total of 108 RAPD markers are generated through 5 primers. Out of these 64 are polymorphic with 59.25% of polymorphism among the taxa studied. The primer OPC-07 has generated maximum per cent of polymorphic bands and least by OPD-02. *C. prasinus* has highest per cent of polymorphism (76.00%) which is a solitary cane and having 3 veined leaflets followed by *C. vattayila* (66.98%) which is also a solitary cane but having 6 veined leaflets. Least per cent of polymorphism was seen in *C. huegelianus* (43.76%), an endangered species. *C. thwaitesii* and *C. huegelianus* are having maximum similarity coefficient of 0.66, whereas *C. vattayila* and *C. nagabettai* show least similarity coefficient of 0.45.

A few studies on phenetic and phylogenetic aspect in rattan species were carried out. Changtragoon et al., [18] carried out a preliminary study on genetic analysis of selected taxa of *Calamus*. 20 primers were screened on the total DNA obtained from the leaf tissue. Only 10 primers yielded reproducible fragments. Out of these, only four primers were selected for further studies. Different populations of *C. thwaitesii* were genetically analysed by Sreekumar & Renuka [2] and concluded that Goa population has a hot spot of genetic variation because of presence of high genetic diversity. Later Sreekumar et al., [19] have reported 95% of polymorphism in *C. rivalis* and *C. metzianus* populations. Based on the RAPD and morphological data, they recommended the merger of these two species. Ramesha et al. [32] have studied the genetic structure of populations of *C. thwaitesii* in core, buffer, and peripheral regions of three protected areas in Western Ghats. Their results indicated that the core and buffer regions maintain a better population's structure as well as higher genetic diversity than the peripheral regions. Sarmah et al. [13] have characterized 15 rattan genotypes using 20 different random primers. Based on RAPD fingerprint, nearly 98.1% of polymorphism was detected among the genotypes. In the present study however per cent of polymorphism in each taxa ranges from 43.67 to 76.00. Further sex identification of *Calamus*

seedlings is a priority because *Calamus* takes 5 years or more to enter the reproductive phase. Molecular markers associated with sex in rattans are needed to identify the sex at an early stage. Li et al. [33] have identified a male-specific SCAR marker (Cs Male 1) in *C. simplicifolius*. While Sarmah and Sarma [34] have identified a female-specific ISSR marker in *C. tenuis*. However, in the present study, seedlings were selected randomly, sex identification studies were not carried out. Such studies are needed in all the five taxa that were selected since they are all economically important and are nearly threatened. This type of studies will definitely help in conservation efforts and breeding programmes. Hemanthakumar et al. [35] have analysed the micropropagated plants of *C. thwaitesii* by ISSR markers and concluded that the microclones are genetically true to their parental origin.

UPGMA cluster analysis of the genetic similarity values has generated a dendrogram illustrating the overall genetic relationships among the taxa studied. The UPGMA cluster analysis assumes a constant evolutionary rate among the genotype and is typically most appropriate for diversity between and within the species. The clustering of the genotypes showed that *C. vattayila* has not clustered with other 4 taxa. Similarly *C. nagabettai* had stood out separately from other three taxa in the cluster 'd'. However, *C. huegelianus* and *C. thwaitesii* form a single cluster. Morphological characters of all the 5 taxa were analysed to study whether their morphological differences are reflected in RAPD profile and dendrogram clustering. Hoey et al. [36] have found close correlation between morphological, isozyme and RAPD data, whereas Khanuja et al. [37], Singh et al. [11] and Joshi et al. [14] have reported the lack of such similarity between morphological and RAPD data. Joshi et al. [14] are of the opinion that as all the loci responsible for expression of morphological traits may not be covered by the molecular markers. This type of analysis in the present investigation, Morphological characters vs. Clustering pattern has led to some conclusion as follows. Formation of main two clusters 'a' and 'b' may be based on leaflet vein characters. Because the two sub groups that are formed under group 'b' which includes taxa that are having 3-veined leaflets, whereas the group 'a' which includes only one taxon, *C. vattayila* has 6 veins in the leaflets. This character has been used as a key character in the dichotomous key framed by Renuka [7]. However *C. huegelianus* and *C. thwaitesii* are clustered with a similarity coefficient of 66. Morphologically both these species are wide apart, each having their own distinguishing characters. Stem is thin about 1.5 cm in diameter in *C. huegelianus* and hence commonly called as 'Pencil cane'. Whereas the stem in *C. thwaitesii* is very stout and about 6 cms in diameter and commonly known as 'Handi betta'. Knee, a swelling of the leaf sheath

below the petiole is present in *C. huegelianus*, whereas it is absent in *C. thwaitesii*. Involucre, the bracteole immediately surrounding the flower, is cup shaped in *C. thwaitesii* and concave in *C. huegelianus*. However, a few characters they share are- leaves are not clustered on the rachis, leaflets are 3 veined; cirrus is absent; both are clump forming. The other taxa which has fallen apart from this group is *C. prasinus*, which is a solitary cane. Though *C. vattayila* also is a solitary cane, it has not grouped with *C. prasinus*. It is completely separated from other taxa, this may be due to the presence of 6 veined leaflets, the characteristic feature, not seen in any other taxa studied. The other taxa which again falls apart in the cluster 'b' is *C. nagabettai*. This taxon exhibits a distinguished morphological character that is the presence of cirrus- an extension of the leaf rachis, armed with reflexed thorns. Cirrus is an important key character in the dichotomous key that is present only in *C. nagabettai*, not in other taxa [7]. The PCA also strengthens the inferences drawn from the cluster analysis of five species of *Calamus*. From the cluster and Principal Component Analysis (PCA) it can be concluded that the samples are clustered together based on sharing of common phenotypic characters. However, a thorough morphological analysis is needed before coming to a conclusion. The highly allogamous nature of the rattan species creates heterogeneity among the species.

Based on the data obtained a dichotomous key was constructed for the selected species.

Dichotomous Key for the selected species of *Calamus*

- | | | |
|-----|--|-----------------------|
| 1a. | Leaflets 6-veined, blade oblong | <i>C. vattayila</i> |
| 1b. | Leaflets 3-veined, blade linear- lanceolate | 2 |
| 2a. | Leaves ending in cirrus | <i>C. nagabettai</i> |
| 2b. | Leaves not ending in cirrus | 3 |
| 3a. | Stem with sheath 3-6 cm in diameter | <i>C. thwaitesii</i> |
| 3b. | Stem with sheath less than 3 cm in diameter | 4 |
| 4a. | Stems solitary. Rachis when cut exudes milky latex , | <i>C. prasinus</i> |
| 4b. | Stems in cluster. Rachis when cut no milky latex, | <i>C. huegelianus</i> |

5. CONCLUSION

Random amplified polymorphic DNA markers were used to evaluate genetic affinities among five economically important *Calamus* species. Based on the scoring of RAPD markers, per cent of polymorphism among the selected taxa is recorded. Thus obtained data was analyzed

using NTSUSpc and UPGMA soft wares and found that the taxa sharing the common phenotypic characters are clustered. Dichotomous key for the selected taxa was proposed based on the results obtained from the present study.

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Explanation to the Figures

Fig. 1. Selected species of *Calamus*

1. *C.nagabettai* 2. *C.thwaitessi* 3. *C.huegelianus* 4. *C.prasinus*
5. *C.vattayila*

Fig. 2. RAPD profiles generated by 5 primers.

Fig. 3. Dendrogram representing simple matching, similarity co-efficient and UPGMA.

Fig. 4. Principal Component analysis of five species of *Calamus*

1. *C.nagabettai* 2. *C.huegelianus* 3. *C.prasinus* 4. *C.thwaitessi*
5. *C.vattayila*