KFRI Project Report No. 436 ISSN 0970-8103

Species recovery of selected endangered rattan species of the Western Ghats

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Kerala Forest Research Institute
An Institution of Kerala State Council for Science, Technology and Environment
Peechi – 680 653, Thrissur, Kerala, India

June 2012

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(Final report of the Project KFRI 567/ '09)

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ABSTRACT OF THE PROJECT PROPOSAL

a. Project number : **KFRI 567/09**

b. Title : **Species recovery of selected endangered rattan species of the Western Ghats**

c. Funding agency : Kerala Forest Department

d. Duration : April 2009-March 2012

e. Objectives:

1. To asses the genetic structure of the populations using molecular markers
2. To identify molecular markers for sex determination
3. To establish seed stands for selected species
4. To enrich the extant populations to create genetically robust populations and to reintroduce the species to other suitable locations

f. Investigators: Dr. E. P. Indira (Objectives 1 & 2)
Dr. C. Renuka (till Sept.2011) (Objectives 3 & 4)
Dr. V.B.Sreekumar (from Sept.2011 onwards) (Objectives 3 & 4)
Research Range officers (Kodanad & Kulathupuzha) ,, ,,

h. Expected outputs:

- The genetic diversity present in the populations can be conserved.
- Seed stands will be established.
- A method will be developed to identify the male and female plants in the seedling stage itself.
- Genetically robust populations will be attained.

Research Fellows: K. Priya and E.L. Linto

ACKNOWLEDGEMENTS

Sincere thanks are due to Dr. K. V. Sankaran, Director, KFRI, for the support and encouragement. We express our gratitude to Kerala Forest Department for financial assistance and all the officials in the Kerala Forest Department for their co-operation during the field work. The hard work provided by Ms. K. Priya, Research Fellow, KFRI, during the laboratory work and the assistance rendered by Mr. E.L. Linto, Research Fellow, KFRI, during the strenuous field work are appreciable and noteworthy. We are grateful to Dr. K.Swarupanandan, Research Coordinator for the support given during the project period. Our sincere thanks are also due to Dr. K.V. Bhat, Dr. R.C. Pandalai and Dr. E.M. Muralidharan for their editorial comments for improving the manuscript.

CONTENTS

	Page
ABSTRACT	1
1. INTRODUCTION	3
2. MATERIALS AND METHODS	9
3. RESULTS AND DISCUSSION	20
4. SUMMARY AND CONCLUSIONS	37
5. LITERATURE CITED	39

ABSTRACT

Over-exploitation of the natural resources along with the destruction of habitat and change of land-use pattern has led to erosion of the diversity of rattans. Hence, conservation of genetic resources is urgently required for which, an understanding of the genetic system and spatial patterns of genetic variation is essential. The genetic diversity in a gene pool is also a major resource to breeders for improvement programme.

This investigation was carried out to reveal the level and partitioning of genetic variation within the four endemic and endangered species, i.e., *Calamus brandisii, C.dransfieldii, C. travancoricus* and *C. vattayila* growing in the Western Ghats. Two types of DNA markers, Random amplified polymorphic DNA (RAPD) and Inter-simple sequence repeat (ISSR), were employed to estimate the genetic diversity of 12 populations belonging to the four *Calamus* species. The results through 20 RAPD and 9 ISSR marker analysis showed that the population of *C. dransfieldii* seen only in Dhoni Forest of Olavakkode Division has high gene diversity. With regard to the two *C. brandisii* populations screened, Shendurney is more diverse than Bonacaud and around 80 percent of the gene diversity resides within populations. The populations are moderately diverse and the genetic differentiation between them is around 20 percent.

Within population gene diversities are low in *C. vattayila* populations, Nelliampathy, Sholayar and Kallar. But, intensity of species level diversity is good enough and genetic differentiation between these populations is very high as 38 to 48 percent indicating the possibility of restricted gene flow or inbreeding. These facts indicate the need for *in situ* conservation of all the populations or collection of germplasm from all the sites for *ex situ* conservation. Dendrogram drawn from the analysis shows that Nelliampathy and Sholayar populations are far distant (though they are geographically closer) than Kallar.

Analysis of six populations in *C. travancoricus* shows that the gene diversity in Kottiyur, Arienkavu and Achenkovil are moderately high while they are low in Shendurney, Sholayar and Mankulam. In this species also, genetic differentiation between populations is high as 30 to 36 percent indicating the likelihood of limited gene flow or mating with relatives. Dendrogram drawn from the data shows a closer relation between Achenkovil and Areinkavu and between Sholayar and Mankulam.

Rattans are dioecious and the sex of the plants cannot be identified till the commencement of flowering. In order to produce sufficient seeds for raising plantations and also for conserving the species, seed stands are necessary. For the production of more seeds, the number of female plants should be more. Hence, for determining the sex of the plants at the seedling stage itself, DNA markers (20 RAPD and 9 ISSR markers) were used. The results show that an RAPD marker, OPAU 02 is efficient to detect female plants in *C. travancoricus*. Likewise, OPAW 20 another RAPD marker is capable of detecting male plants in *C. brandisii*. Using the results from the present study, development of scar markers for sex determination in these two species is possible. The study also indicates the possibility of hermaphrodite flowers/ plants in *Calamus* sp. which should be ascertained through further field studies.

As envisaged, efforts were made to establish seed stands. One thousand seedlings of *C. vattayila* and *C. travancoricus*, originated from nine populations from Kerala, were field planted in Malayattur Division. At the age of two years, 65- 75 % of the seedlings survived and the rest were destroyed mostly by wild pigs. For enriching the existing populations to create genetically robust populations, populations having high gene diversity were identified. Seedlings originated from different populations of the four species were raised in the nursery and a total of 1500 seedlings of *C. vattayila* and 500 seedlings of *C. travancoricus* were handed over to the Research Range Office, Kodanad to enrich the extant populations. Seedlings of all the four species were planted in the KFRI arboretum and representative samples were added to the Palmetum.

1. INTRODUCTION

Rattans are a group of spiny climbing palms with about 600 species in 13 genera distributed worldwide which show great variation in morphology, ecology, altitudinal preferences, geographical distribution and end uses. Rattans are also referred to as 'green gold' for their unique characteristics including strength, durability and flexibility. It is an important raw material for cane furniture and handicrafts industries. Dransfield and Uhl (1986) have placed rattans under a distinct subfamily *Calamoideae* of the family *Arecaceae (Palmae)*. "Rattan", is an English word derived from a Malayan word "rotan" which is a collective name of a vast group of palms meaning 'scaly fruited' in Greek. Rattans are one of the most important non-wood forest products distributed in equatorial Africa, Southern China, the Malay Archipelago, Australia and the Western Pacific. They are distributed mainly in the evergreen, semi-evergreen and moist deciduous forests. Some species occur in sacred groves also. Rattans grow at elevations ranging from sea level to 2000m above msl, most species showing altitudinal preferences. Majority of the species are distributed at altitudes below 1000m. Six species are seen only at elevations above 700m.

In India, there are about 61 species of rattans under four genera namely *Calamus, Daemonorops, Korthalsia and Plectocomia* (Renuka, 1999) distributed in three major eco-geographic regions; Peninsular India, North and North Eastern India and Andaman and Nicobar Islands. Kerala is represented with one genus *Calamus* with 17 species. Basu (1985) reported that out of the four genera of rattans occurring in India, only one genus, viz. *Calamus* is found in South India.

It is estimated that around 117 species of rattans are treated as threatened to some degree (Walter and Gillet, 1998). Habitat destruction poses a more permanent and widespread threat to palms in the tropical forests. The two main threats to rattans are overexploitation and habitat destruction. Species, whose habitat range is limited to a small area, are mostly at risk.

Most of the rattans in Kerala are 'clump forming' or 'clustering' (multi-stemmed) except *C. dransfieldii, C. delessertianus* and C. *vattayila* which are 'solitary' (single stemmed) climbers.

Calamus species are dioecious, i.e. the male and female flowers are borne on separate plants. In *Calamus subinermis,* separate male, female and hermaphrodite plants were reported (Chia, 2000). In few plants both male and hermaphrodite flowers also were seen.

Rattans are exploited for their flexible stems, the cane providing the raw material necessary for cottage industries, furniture, for the manufacture of handicrafts and other utility items like baskets, walking sticks and mats. Rattans are useful for different purposes because they are light and strong with smooth surface. They are one of the non wood forest products, which provide livelihood for millions of tribal and rural people and raw materials for industries in various parts of the world.

The diameter of stem varies from 3mm to 30mm depending on the species. In many species the stem is very long and at times more than 90m. Stem surface is smooth and straw yellow in colour. Canes develop a good luster when rubbed with sand and coconut husk and washed in water before drying. Rattans are popular because of their durability, elasticity, light-weight, lustrous-brown colour and flexibility, which allow them to bent and molded in different shapes and designs. Rattans are also used for preparation of sports goods like javelins, cricket bats and hockey sticks.

Apart from their importance as a commodity for the furniture making industries, in ancient times, medicinal uses of rattan have also been reported. *Calamus rotang,* in combination with other herbs, is useful for treating snake bites and scorpion stings. In veterinary practices, it is employed as tonic and aspirant. The tender leaves of *Calamus travancoricus* are used for treating dyspepsia, ear trouble and are also anthelmintic. The tribal people make use of long canes for making cane bridges.

Increase in demand for the raw material has resulted in over-exploitation of the natural resources. This, along with the destruction of habitat and change of land-use pattern has led to erosion of the diversity of rattans. Many of the species have become rarer in distribution and are threatened with extinction. Hence, conservation and utilization of the genetic resources of rattans require urgent action. Conservation measures require an understanding of the genetic system and spatial patterns of genetic variation. Habitat preservation in the form of National Parks and strict nature reserves can lead to *in situ* conservation. For *ex situ* conservation large scale plantation programme should be implemented. Reforestation projects may also be adopted.

Raising plantations require quality planting stock in a large scale. Seed is the best source of propagation but its large scale availability is a problem in the present situation. Hence, as a first step, seed orchards should be developed to ensure a steady supply of seeds for the future plantation programmes. Kerala Forest Research Institute has already raised seed stands of four commercially important species of rattans. The nursery and planting techniques have also been standardized (Renuka, 1990). Ecosystem conservation together with large scale plantation of rattans will guarantee the conservation of the species and genetic diversity.

Genetic diversity plays an important role in any of the breeding/improvement programme. The more the variation, the better the chance that at least some of the individuals will have an allelic variant that is suited for the new environment and they will produce offspring with the variant that will, in turn, reproduce and continue the population into subsequent generation. In order to develop scientifically sound, comprehensive and resource effective strategy for gene pool conservation, knowledge of the pattern of distribution of genetic diversity in space and time is necessary.

Molecular markers are rapidly being developed in recent years for tree improvement and increasingly used for diversity analysis, germplasm characterization and identification of core population. These biotic tools are useful in planning proper conservation strategies and also to carry out molecular breeding amongst elite trees to

produce superior clones (Beckman and Soller, 1990). DNA markers assumed importance since they can be used as genetic markers that are associated with economically important traits (Beckman and Soller, 1990). DNA markers, because of their heritable nature, were found to act as versatile tools in the fields of taxonomy, physiology, embryology and genetic engineering. They are used for detecting variations in DNA that differentiate all living organisms and thus play an important role in defining biological identity.

Inter simple sequence repeats (ISSR) are arbitrary multiloci markers produced by amplification with microsatellite primers. In this technique, primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. These are mostly dominant markers, though occasionally a few of them exhibit co-dominance. ISSR markers are used in population genetic studies and also in detecting clonal diversity and in resolving taxonomic relationships at or below the species level. They produce more reliable and reproducible bands than RAPDs because of the higher annealing temperature and longer primer sequences. ISSR primers anneal to simple sequence repeats that are abundant throughout the eukaryotic genome and evolve rapidly, hence, may reveal a high level of polymorphism.

Randomly amplified polymorphic DNA (RAPD) is a PCR based molecular marker technique developed by Williams *et al.* (1990). In this technique, single short oligonucleotide primers are selected to amplify a set of DNA segments distributed randomly throughout the genome. Because these primers are 10 nucleotides long, they have the possibility of annealing at a number of locations in the genome. They are dominant markers, hence do not permit the scoring of heterozygous individuals. They are useful for efficient screening of nucleotide sequence polymorphism between individuals. RAPD is technically simple, requires only nanogram amounts of DNA and reveals high levels of sequence polymorphism. RAPDs have been used as an efficient method to reveal genetic structure and diversity patterns of plants and are useful for DNA fingerprinting

A few studies based on DNA analysis were undertaken in *Calamus* species by various organizations. As a part of the EU-funded rattan project, Bon *et al*. (1996) investigated genetic variability in *Calamus subinermis*. They demonstrated considerable variability in wild populations of this species in Sabah and also showed that the variability had some correlation with geographic distribution, with populations occurring near to each other being more closely related genetically than those occurring further in the field.

Assessment of genetic diversity in *Calamus thwaitesii* Becc. (Arecaceae) using RAPD markers (Sreekumar and Renuka, 2006) was carried out at KFRI and the authors report that majority of the genetic diversity was distributed within the populations (70.79%) and only 29.21% among populations.

Taxonomic reconsideration of *Calamus rivalis* and *C. metzianus* was attempted through morphometric and molecular analyses at KFRI. Analysis of different Indian and Sri Lankan populations of these two rattan species was carried out using RAPD markers and morphological characters revealed the existence of only a single species and suggested merging of *C. rivalis* with *C. metzianus* (Sreekumar *et al*., 2006).

The RAPD marker technique was used to determine the sex of *Calamus simplicifolius* (Yang *et al*., 2005) where the authors could identify a male spcific marker which helped in the identification of the sex at the early stages of plant life, which is useful for breeding programs. Thirty ISSR primers were screened on female and male *Calamus tenuis* plants from five different provinces of Assam, India. A putative female-specific marker was identified which is useful in genetic improvement of the species (Sarmah and Sarma, 2010).

Previous demographic and reproductive biological studies on different species (Anto *et al*., 2008, Renuka and Rugmini, 2007) showed that certain rattan species in the Western Ghats have been reduced to incredibly low populations due to several reasons. These studies reported that the populations of *Calamus brandisii*, *C.*

vattayila and *C. travancoricus* are declining in the natural forests. For *C. brandisii* in Agstyamalai region, especially on the way to Agastyakoodam, there was a decrease of 61 per cent during the years 2004-2007. There is an annual decrease of nine per cent for *C. travancoricus* and six per cent for *C. vattayila.* Another endangered rattan, *C. dransfeildii*, endemic to Dhoni forests, also shows a decreasing trend. Here the population is very small and only a few individuals are present in this area. All these species are good quality canes used extensively in furniture and handicraft industries. It is feared that without immediate attention, some of these species may become extinct. Hence, conservation measures are the need of the hour as far as these species are concerned.

Sufficient quantities of seeds are not available for the above species. Hence, seed stands should be established for the above mentioned species for future plantation efforts. Rattans are dioecious and the sex of the plant cannot be identified till flowering starts. More female plants are required to produce enough seeds for the next generation. Hence, methods should be developed for the determination of sex of the plant in the seedling stage itself during the establishment of the seed stands.

Considering the above facts, the present study was undertaken for the recovery of four endangered rattan species, *C. brandisii, C. dransfieldii, C travancoricus* and *C. vattayila* with the following objectives.

Objectives:

1. To assess the genetic structure of the populations using molecular markers
2. To identify molecular markers for sex determination
3. To establish seed stands for selected species
4. To enrich the extant populations to create genetically robust populations and to reintroduce the species to other suitable locations

2. MATERIALS AND METHODS

The populations of four endangered rattan species, *C. dransfieldii*, *C. brandisi*, *C. vattayila* and *C. travancoricus* discussed in the present study were selected at different localities in various forest divisions in Kerala (Table 1, Fig.1). A view of the four species selected is given in Plate 1.

***Calamus dransfieldii* Renuka** is endemic to Dhoni hills of Palakkad in Olavakkode Division. The species is very patchy with very few plants present in this locality. It is a good, large diameter cane used in furniture industry and in basket making. They occur as both solitary or clustered, moderate-sized canes, climbing high up into the canopy (Plate 1). Their stem with sheaths is about 3.5 cm in diameter, without sheaths about 2.5 cm. Leaves are about 2m long; leaf sheath pale green, sparingly spiny with bulbous based spines; knee conspicuous; leaflets pale green, regularly arranged, about 45 x 2 cm, veins ciliate on both surfaces; cilia to 2 cm long. Inflorescences are long, flagellate, partial inflorescence to 9 cm long; fruits to 2.5 cm across, globose.

***Calamus brandisii* Becc.** is distributed in the evergreen forests between 1000-1500m at Bonacaud and Agasthyamala, (Thiruvananthapuram District) and at Pandimotta (Kulathupuzha) in Kerala and at Kalakkadu and upper Kothayar in Tamil Nadu. It is an excellent small diameter cane, extensively used in furniture industry. It is a small diameter clustering type cane. With respect to the morphological characters, its stem is with sheaths to 1.5 cm in diameter, without sheaths up to 0.8 cm. Leaves about 1m long; leaf sheath green, with minute bristle like spines; mouth of the sheath with larger spines to 4 cm long; knee present; leaflets grouped. Male and female inflorescences are long and slender. Fruits are ovate, 1.8 x 0.8 cm, scales arranged in 17 vertical rows, slightly channelled in the middle, brown with dark brown border.

***Calamus vattayila* Renuka,** is seen sporadically in evergreen forests between 200 and 750m altitude. This is reported from forests of Thenmala, Ranni, Nilambur, Wayanad, Nenmara, Thekkady, Chalakudy and Vazhachal. The distribution extends

to Tamil Nadu and Karnataka. Their population size is small and the plants are seen scattered. It is a single stemmed medium sized good quality cane, mainly used in furniture industry. This good quality cane used as round and split forms in chair/seat backs; is also used for walking sticks/umbrella handles, basketry and handicraft items. The restricted distribution and the overexploitation by the furniture industry have made this species endangered. The local name "vattayilayan" comes from the shape of the leaflet. Its stem is with sheaths up to 5 cm in diameter at apex and 2.5 cm at base, without sheaths 1.8 cm. Leaves 1m long; leaf sheath dark green and sparingly spiny; spines generally pointing upwards; leaflets alternate, about 40 x 10 cm; inflorescence to 1m long; partial inflorescence to 40 cm long; getting shorter towards the tip of the inflorescence. Fruits are in heavy bunches; a single fruit measuring about 2.5 x 0.8 cm, oblong, scales in 27 rows, longer than broad, chestnut brown coloured.

***Calamus travancoricus* Bedd. ex Becc. & Hook.f.** (Local name -arichural, kiri betha) is seen only in the evergreen forests from 200-500m in Thiruvananthapuram, Thenmala, Ranni, and konni, Malayattoor, Chalakudy, Vazhachal and Nilambur Forest Divisions of Kerala. It is a good quality small diameter cane used extensively in handicraft and furniture industries, but not available in sufficient quantities. It is a clustering, very slender climbing cane. With respect to the morphological characters, Stem is with sheaths up to 0.8 cm in diameter and without sheaths to 0.4 cm. Leaves are to 45 cm long; leaf sheaths green, armed with small spines of 0.5 cm length, mouth of the sheath with slightly longer spines of 0.75 to 1cm length; leaflets grouped along the rachis. Inflorescences are about 1m long, partial inflorescence 10-12 cm long. Fruits are 1cm across, globose, scales in 24 rows, straw yellow with a dark brown border.

SAMPLE COLLECTION

Fresh leaf samples were collected from the one and only population of *C. dransfieldii* at Dhoni Hills, from two populations of *C. brandisii* at Bonacaud and Shendurney, from three populations of *C. vattayila* from Nelliampathy, Kallar (Achenkovil) and

Sholayar regions and from six populations of *C. travancoricus* from Achenkovil, Arienkavu, Kottiyur, Sholayar, Shendurney and Mankulam (Table 1, Fig.1). Seeds were also collected from the above locations for establishing seed stands and to enrich extant populations.

Plate 1. A. *Calamus brandisii* **Becc. B.** *Calamus dransfieldii* **Renuka C.** *Calamus travancoricus* **Bedd. D.** *Calamus vattayila* **Renuka**

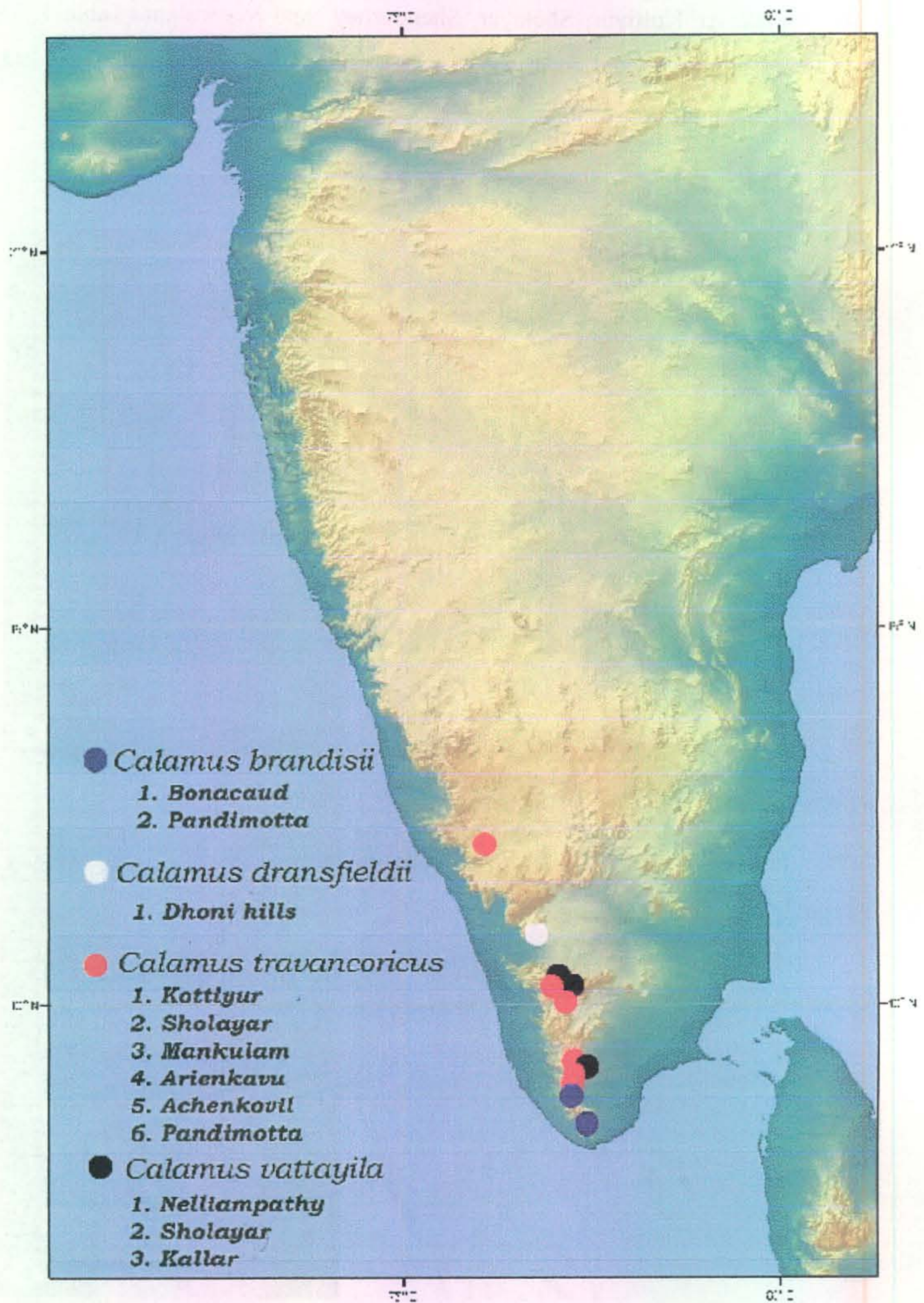


Fig.1. Populations selected for the DNA marker study

Table 1. Populations selected for gene diversity

Sl. No.	Calamus species	Populations & Forest Division	Latitude (N)	Longitude (E)	No. of samples
1	*C. dransfieldii*	Dhoni hills Palakkad Division	10° 51'N	76°37' E	15
2	*C. brandisi*	Bonacaud, Peppara Wildlife Sanctuary,	8°45'N	77°11'E	30
		Pandimotta, Shendurney Wildlife sanctuary,	8°81'	77°23'	10
3	*C. vattayila*	Nelliampathy, Nenmara Forest Division	10°41'	76°39'	30
		Kallar, Achenkovil	9°14'	76°46'	20
		Sholayar, Vazhacahal Division	10°21'	75°52'	6
4	*C. travancoricus*	Kottiyur, Kannur Dvsn.	11°53'	77°23'	30
		Sholayar, Vazhacahal Division	10°21'	75°52'	10
		Pandimotta, Shendurney Wildlife Sanctuary,	8°81'	77°23'	7
		Achenkovil	9°14'	76°46'	10
		Arienkavu, Thenmala Division	9°01'	77°08'	10
		Mankulam, Mankulam	10°07'	76°56'	10

I. Genetic structure of populations

DNA EXTRACTION

The leaf samples were immediately transferred to CTAB buffer and stored at -20°C until DNA was extracted. Genomic DNA was isolated from 1g of fresh leaf tissue of individual plants using the slightly modified CTAB method (Doyle and Doyle, 1987).

For DNA extraction initial grinding with liquid nitrogen was employed to break down the cell wall. Once the tissue was sufficiently ground, it was suspended in CTAB. In order to purify DNA, insoluble particulates were removed through centrifugation while soluble proteins and other materials were separated through

mixing with chloroform followed by centrifugation. DNA was precipitated from the aqueous phase which was washed thoroughly to remove contaminating salts. The purified DNA was then re-suspended and stored in TE buffer or sterile distilled water. 3µl of RNase (10mg/ml) was added and incubated at 37°C for 30 minutes for immediate analysis or stored at -20°C.

For gender identification or sex determination, DNA was also isolated from 10 male and 10 female plants of *C.brandisii* and *C.travancoricus* and re-suspended in 100uL TE buffer.

DNA QUANTIFICATION AND AGAROSE GEL ELECTROPHORESIS

The concentration of DNA in the samples was estimated using the Nanodrop Quantifier (Nanodrop 1000 Spectrophotometer Thermo Scientific).

The presence of genomic DNA from plants was checked in 0.8% agarose gel. The DNA samples were mixed with loading dye (Bromophenol blue) and loaded into the gel. The gel was run at 50V for 20 minutes and the presence of intact DNA bands was confirmed. The DNA was visualized in the gel by the addition of Ethidium bromide which binds strongly to the DNA by intercalating between the base pairs.

DNA AMPLIFICATION THROUGH POLYMERASE CHAIN REACTION

The total genomic DNA isolated from the four *Calamus* species was PCR amplified using 20 RAPD (Randomly Amplified Polymorphic DNA) markers (OPA Kit, Sigma, USA) and 10 ISSR (Inter Simple Sequence Repeats) markers (UBC Series, Sigma, USA) to assess the genetic diversity within and among the populations.

Primer screening: RAPD primers

A total of 40 RAPD primers from the OPA kit were screened on the total DNA extracted from the leaf tissues of *C. dransfieldii*, *C. brandisii*, *C. vattayila* and *C. travancoricus*.

Among these, only 20 primers yielded good reproducible amplification products and were selected for the RAPD analysis. The 20 amplified RAPD primers used in the analysis are listed in Table 2. PCR reactions were performed on a PTC-200 thermocycler (MJ Research Inc, USA) in 25 μL reaction mixtures containing 25 – 50 ng of template DNA, 1mM of dNTP mix, 5pM of primer, 1.5 unit of Taq DNA polymerase (Genei, Bangalore), 10mM $MgCl_2$ and 10X Taq buffer with 15mM $MgCl_2$. The amplification was programmed with an initial denaturation of 95°C for 4 minutes and 45 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes. The last cycle was followed by a final extension of 72°C for 4 minutes.

Table 2. Details of selected RAPD primers

Sl No.	RAPD Primers	Sequence 5'-3'
1	OPA-03	AGTCAGCCAC
2	OPA-04	AATCGGGCTG
3	OPA-09	GGGTAACGCC
4	OPA-11	CAATCGCCGT
5	OPA-12	TCGGCGATAG
6	OPA-13	CAGCACCCAC
7	OPA-15	TTCCGAACCC
8	OPA-16	AGCCAGCGAA
9	OPA-17	GACCGCTTGT
10	OPA-18	AGGTGACCGT
11	OPA-20	GTTGCGATCC
12	OPA-10	GTGATCGCAG
13	OPB-15	GGAGGGTGTT
14	OPE-02	GGTGCGGGAA
15	OPE-18	GGACTGCAGA
16	OPAU-02	CCAACCCGCA
17	OPAW-07	AGCCCCCAAG
18	OPAW-09	ACTGGGTCGG
19	OPAW-10	GGTGTTTGCC
20	OPAW-20	TGTCCTAGCC

ELECTROPHORESIS OF THE AMPLIFIED PRODUCTS:

RAPD products were electrophoresed on 1.2% agarose gel using 1X TBE buffer at a constant voltage of 55Volts for 2½ -3 hours and the bands were compared with 100/250bp DNA ladder. The gel was visualized under the Electronic UV Trans-illuminator by Ultra CAM Digital Imaging System.

Primer screening: ISSR primers

To identify the primers that detect polymorphism, a total of 20 ISSR primers were screened on the total DNA extracted from the leaf tissues of all the species. From these, only nine primers yielded good reproducible amplification products and were selected for the ISSR analysis (Table 3). PCR reactions were performed in 25 μL reaction volumes containing 10X Taq buffer with 15mM $MgCl_2$, 50 ng of template DNA, 10mM $MgCl_2$, 1mM of dNTP mix, 10 picomoles of primer, 2 units of Taq DNA polymerase, using PTC 200 Thermal Cycler. The amplification reactions were carried out with an initial denaturation of 94°C for 5 minutes and 30 cycles for 45 seconds of denaturation at 94°C, 45 seconds of annealing at 52°C, and 120 seconds of extension at 72°C. The last cycle was followed by a final extension of 72°C for 8 minutes.

Table 3. Details of selected ISSR primers used in the analysis

Sl. No.	ISSR Primers	Sequence (5'-3')
1	UBC 834	AGAGAGAGAGAGAGAGYT
2	UBC 835	AGAGAGAGAGAGAGAGYC
3	UBC841	GAGAGAGAGAGAGAGAYC
4	UBC855	ACACACACACACACACYT
5	UBC868	GAAGAAGAAGAAGAAGAA
6	UBC880	GGAGAGGAGAGGAGA
7	UBC890	VHVGTGTGTGTGTGTGT
8	ISSR 4	AAGAAGAAGAAGAAGCC
9	ISSR 5	AGCAGCAGCAGCAGCCA

Key to symbols : Y = C + T , V = G + A + C, H = A + T + C ,

ELECTROPHORESIS OF THE AMPLIFIED PRODUCTS:

The ISSR amplified products were separated on 1.5% agarose gel using 1X TBE buffer at constant voltage of 55 Volts for 2-3 hours and the bands were compared with 250 base pairs DNA ladder. The gel was visualized under the Electronic UV Transilluminator by Ultra CAM Digital Imaging System.

II. Identification of molecular markers for sex determination

Isolation of genomic DNA

Ten male and ten female plants of C. *brandisii* were collected from the Bonacaud population (Peppara Wildlife Sanctuary) and ten male and female plants of *C. travancoricus* from Achenkovil and Arienkavu populations. The leaf materials were collected from mature plants of *C. brandisii* during flowering (October-December) and fruiting seasons (March-May). In *C. travancoricus* leaf samples were collected during the flowering in October-November and fruiting during May-June. The plants that bear seeds were tagged as female and the plants that bear male inflorescence were tagged as male. The leaf specimens were stored at -20°C until the DNA was extracted.

PCR ANALYSIS

RAPD AND ISSR ANALYSIS

As explained earlier DNA samples were extracted separately. The selected 20 RAPD primers and 9 ISSR primers were used for the DNA profiling as noted earlier.

ALLELE SCORING

According to the position of bands, loci were differentiated for each marker. The shortest fragment moved extreme downwards. Bands of identical size amplified with the same primer were considered to be the same locus consisting of two alleles. Bands were scored based on the presence (taken as 1) or absence (taken as 0) at each locus for all the primers. Only clear and recognizable bands were scored. The size of

the amplified bands was determined based on its migration relative to the molecular size marker (DNA Ladder).

STATISTICAL ANALYSIS

Amplification with each primer with clearly resolved reproducible fragments were considered in data collection. Amplified fragments were scored as binary data, i.e., presence as 1 and absence as 0, for the homologous bands. The data matrices were entered into the PopGene Version 1.31 package and pair-wise comparison of populations was made. The 1/0 matrix was prepared for all the fragments scored and was used to calculate pair wise genetic distances following Nei and Li (1979). The matrix of RAPD phenotypes was analyzed on the basis of several indices of population genetics, such as number and percentage of polymorphic loci, observed number of alleles, effective number of alleles, Nei's gene diversity (*h*) and Shannon's information index (*I*) using POPGENE program version 1. 31 (Yeh *et al.*, 1999). Nei's unbiased genetic distances (1978) were calculated among and between the populations i.e. within species diversity (Hs) and total genetic diversity (Ht) (Nei, 1978) were calculated. The matrices of Nei's genetic distances were used to cluster the populations by the UPGMA method (Unweighted pair group method using arithmetic average) using the software package POPGENE version 1.21 (Yeh and Boyle, 1997).

III. & IV. Establishment of seed stands and reintroduction of the species to other suitable locations

Field surveys were conducted throughout the geographical ranges of *C. brandisii*, *C. dransfieldii*, *C. travancoricus* and *C. vattayila*, and specimens and leaf samples were collected. All the voucher specimens were deposited in KFRI herbarium.

Mature fruits were collected from different locations except for *C. dransfieldii*. *C. dransfieldii* is endemic to Dhoni forests and the fruit set and regeneration status was extremely low in its natural sites. The collected fruits were pretreated in fresh

water for 48 hours, peeled to extract the seeds and were put to germination in the nursery beds. Seeds of each population were kept separate. Germinated seeds from the nursery beds were transplanted to polybags of 23 x 15 cm size, filled with forest topsoil and sand in a ratio of 3:1. Immediately after transplanting, these polythene bags were shifted to the nursery under semi-shaded area (about 50% ambient light intensity) and watered every day to ensure adequate moisture. Weeding was carried out at regular intervals till out planting. All the seedlings were raised in the nursery at KFRI.

For establishing seed stands, one-year-old seedlings (1000 Nos.) were handed over to the Research Range Officer for planting in forest areas at Kodanad, Malayattur Forest Division. The details of seedlings planted for establishing seed stands is given in Table 8. The planting was done after the pre-monsoon showers in a randomized complete block design (RCBD) with equal replications in a block. The survival rates of the seedlings were recorded.

In order to enrich the existing populations, 1500 seedlings of *C. vattayila* and 500 seedlings of *C. travancoricus* were handed over to the Research Range Office, Kodanad.

3. RESULTS AND DISCUSSION

I. Genetic structure of the populations

Among the 40 RAPD primers, only 20 primers yielded good reproducible amplification products and out of the 20 ISSR primers only 9 primers have given good amplification. Pictures of the gels are shown in Fig.2 and 3.

Fig. 2 Picture of the gel (RAPD)

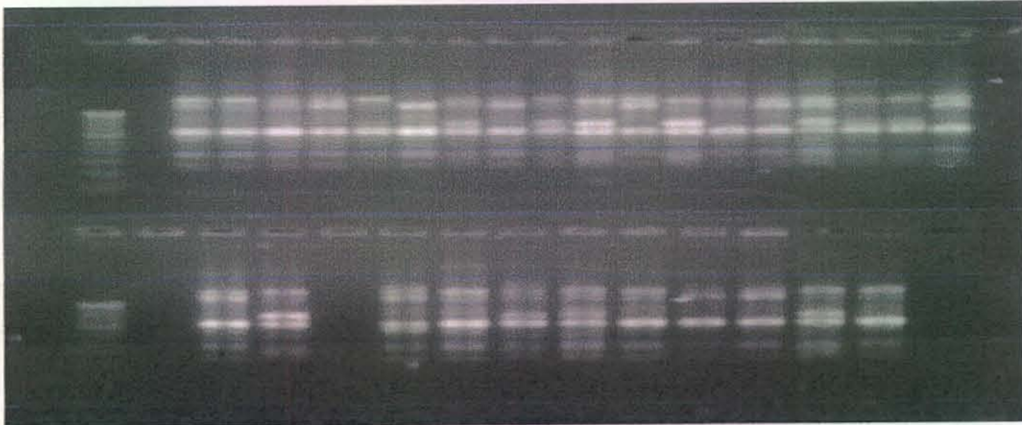
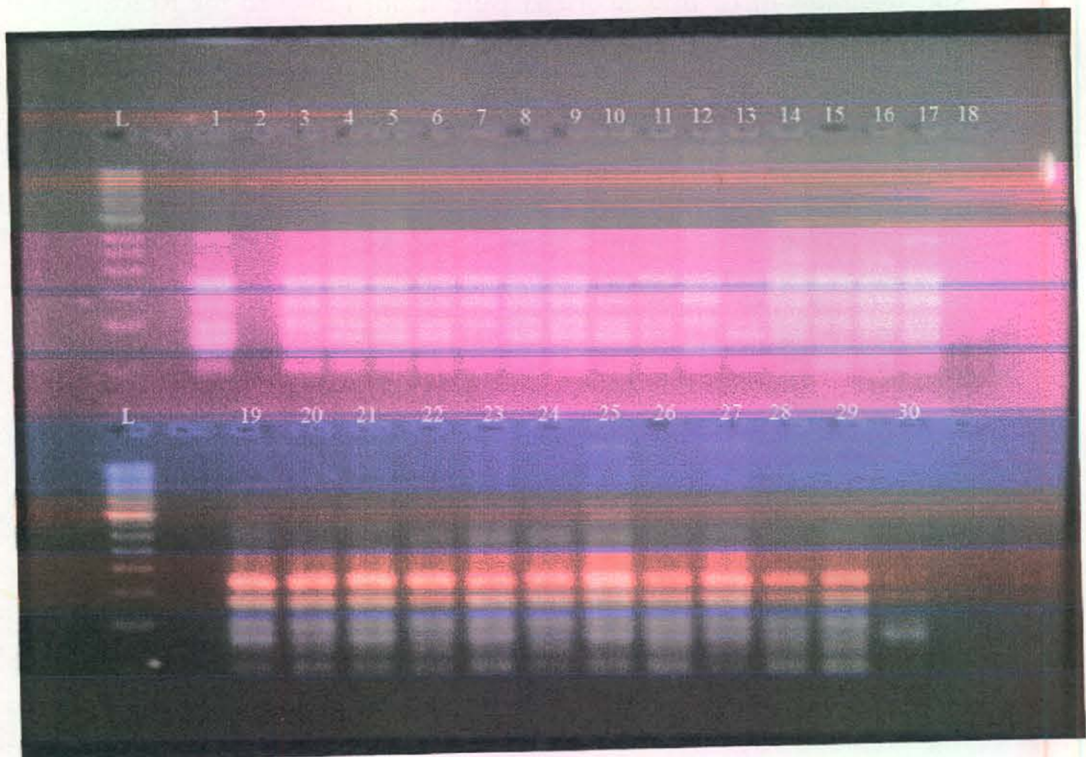


Fig. 3 Picture of the gel
(ISSR)



The results of the analysis including the genetic parameters are given in Tables 4 and 5 with respect to all the four species.

C. dransfieldii

C. dransfieldii is seen only in the Dhoni forests as noted earlier and hence, only one population was analysed.

ISSR markers

With respect to 9 ISSR markers used in the present studies, a total of 38 fragments were scored of which, 25 were polymorphic. The percentage of polymorphism generated for all primers was 65.79%. Number of bands varied from three (UBC 880) to five (UBC 855, UBC 868 and ISSR 5). The band size generated by the nine primers ranged from 250 to 2000 base pairs. Out of an average of 1.658 alleles, 1.543 were effective alleles. The gene diversity was found to be 0.295 which is quite high.

RAPD markers

20 RAPD markers employed in the present studies generated a total of 73 fragments of which, 48 were polymorphic fragments. The percentage of polymorphism was 65.75%. Number of bands varied from two (OPE 02 and OPAW 07) to six (OPA 10 and OPA 13).The band size generated by the twenty primers ranged from <250 to 2500 base pairs. Out of an average 1.658 alleles, 1.55 were effective alleles. The gene diversity was found to be 0.297 which is quite high (Tables 4 and 5).

Both of the markers, ISSR and RAPD, showed a similar pattern of polymorphism (65.75 and 65.79%). For both the markers average number of alleles was 1.658, of which 1.543 (for ISSR) and 1.55 (for RAPD) were effective alleles. Moreover, the gene diversity exhibited by the markers was 0.295 and 0.297 respectively with ISSR and RAPD markers. The percentage of polymorphism and gene diversity are quite comparable with the mean of many the other species (P=64.7% and H= 0.257) (Hamrick and Godt, 1989).

C. brandisii

Two populations, Bonacaud and Shendurney were analysed for the genetic parameters.

ISSR markers

Nine ISSR markers used in the Bonacaud population showed a total of 46 fragments of which, 23 were polymorphic fragments. The percentage of polymorphism was 50%. The band size generated by the primers ranged from 250 to 1750 base pairs. The results are presented in Table 4. Out of an average of 1.5 observed alleles, 1.33 were effective alleles. The gene diversity was found to be 0.194 in Bonacaud showing the genetic diversity to be low.

In the population from Shendurney (Pandimotta), a total of 46 fragments were scored of which, 33 (71.74%) were polymorphic. Out of an average 1.72 observed alleles, 1.61 were effective alleles. The gene diversity was found to be 0.327 which is quite high compared to Bonacaud and many other populations and species.

When the two populations, Bonacaud and Shendurney (Pandimotta), were taken together, the value for total genetic diversity among populations (Ht) was 0.326 while within population diversity (Hs) was 0.261. The percentage of total genetic differentiation (19.9%) among the two populations (Gst= 0.199) was relatively higher than the mean *Gst* value (0.129) reported in many species (Hamrick *et al.*, 1992). These results showed that 80.1% of the genetic diversity resided within the populations indicating that there are higher variations within the population. This is useful in making strategy for germplasm collection and evaluation. Gst of 19.9% is between the average of out crossing plant species (*Gst* = 10%) and that of selfing plant species (*Gst* = 51%) (Hamrick and Godt, 1989).

RAPD markers

Twenty RAPD markers used in the Bonacaud population resulted in a total of 99 fragments of which, 62 (62.63%) were polymorphic. The band size generated by

the nine primers ranged < 250 to 2500 base pairs. Out of an average 1.63 observed alleles, 1.45 were effective alleles. The gene diversity was found to be 0.258 in Bonacaud which is a moderate value.

In the population from Shendurney (Pandimotta), a total of 99 fragments were scored of which, 56 (56.57%) were polymorphic. On an average 1.43 alleles were effective out 1.57 observed alleles. The gene diversity was found to be 0.236 which shows the population to be moderately diverse.

When the two populations, Bonacaud and Shendurney, were taken together, the value for total genetic diversity among populations (Ht) was 0.318 while within population diversity (Hs) was 0.247. The percentage of total genetic differentiation (22.3%) among the two populations (Gst= 0.223) is relatively higher than the mean *Gst* value (0.129) reported in many other species (Hamrick *et al.*, 1992). This result indicated that 77.7% of the genetic diversity resided within the populations.

The studies also show that when ISSR markers were employed, genetic diversity was high in the population from Shendurney (0.327) while it was low in Bonacaud (0.194). Shendurney can be considered as an important population of high gene diversity with potentially useful genes and may be included in the high priority reservoir for genetic conservation. But when RAPD markers were used, both Bonacaud population (0.258) and Shendurney (0.236) were moderately diverse. The genetic distance between these two populations was 0.1739 with ISSR markers and 0.1971 with RAPD markers.

The marked difference in estimation of gene diversity in the same population at Bonacaud when two markers were employed is noteworthy. However, ISSR markers help to analyse the conserved (may be coding or non coding) or nonconserved region, while RAPD markers help to analyse the diversity largely in the noncoding regions.

Table 4. Polymorphism and gene diversity in *Calamus* sp. with ISSR markers

Species &Population	No. of polymorphic loci	% polymorphic loci	No. of alleles	No. of effective alleles	Gene diversity (species level)	Gene diversity (Hs) in sub-population	Gene diversity (Ht) in total population	Genetic differentiation (Gst)
1. C. dransfieldii								
1.Dhoni (15)	25	65.79	1.658	1.543	**0.295**	**0.295**		
2. *C. brandisii*								
1.Bonacaud (30)	23	50	1.5	**1.33**		**0.194**		
2.Kulthpuzha(10)	33	71.74	1.72	**1.613**		**0.327**		
With respect to all sub-populations	**36**	**78.26**	**1.783**	**1.468**	**0. 276**	**0. 261**	**0. 326**	**0. 199 (19.9%)**
3. *C. vattayila*								
1.Nelliamp (30)	19	41.3	1.413	**1.275**		**0.16**		
2.Kallar (20)	18	39.13	1.391	**1.299**		**0.163**		
3.Sholayar (6)	13	28.3	1.283	**1.211**		**0.118**		
With respect to all sub-populations	**29**	**63.04**	**1.63**	**1.365**	**0.214**	**0.147**	**0.238**	**0.383 (38.3%)**
4. *C. travancoricus*								
1.Kottiyur (30)	28	56	1.56	**1.37**		**0.218**		
2.Sholayar (10)	19	38	1.38	**1.29**		**0.161**		
3.Kulathupzh (7)	22	44	1.44	**1.39**		**0.204**		
4.Achenkvil (10)	26	52	1.52	**1.39**		**0.219**		
5.Arienkav (10)	30	60	1.6	**1.43**		**0.238**		
6.Mankulam(10)	16	32	1.32	**1.27**		**0.145**		
With respect to all sub-populations	**43**	**86**	**1.86**	**1.486**	**0.285**	**0.197**	**0.281**	**0.298 (29.8%)**

Table 5. Polymorphism and gene diversity in *Calamus* sp. with RAPD markers

Species &Population	No. of polymorphic loci	% polymorphic loci	No. of alleles	No. of effective alleles	Gene diversity (species level)	Gene diversity (Hs) in sub-population	Gene diversity (Ht) in total population	Genetic differentiation (Gst)
1. *C. dransfieldii*								
1.Dhoni (15)	48	65.75	1.658	1.55		**0.297**		
2. *C. brandisii*								
1.Bonacaud (30)	62	62.63	1.626	1.453		**0.258**		
2.Kulthpuzha(10)	56	56.57	1.566	1.434		**0.236**		
With respect to all sub-populations	**80**	**80.81**	**1.808**	**1.526**	**0. 306**	**0. 247**	**0. 318**	**0. 223 (22.3%)**
3. *C. vattayila*								
1.Nelliamp (30)	50	41.7	1.417	1.271		**0.157**		
2.Kallar (20)	64	53.3	1.533	1.376		**0.213**		
3.Sholayar (6)	39	32.5	1.325	1.257		**0.14**		
With respect to all sub-populations	**104**	**86. 67**	**1. 867**	**1. 582**	**0. 331**	**0. 17**	**0. 331**	**0. 486 (48.6%)**
4. *C. travancoricus*								
1.Kottiyur (30)	70	67.96	1.68	1.47		**0.266**		
2.Sholayar (10)	47	45.63	1.46	1.36		**0.194**		
3.Kulathupzh (7)	34	33.01	1.33	1.26		**0.142**		
4.Achenkvil (10)	60	58.25	1.583	1.433		**0.241**		
5.Arienkav (10)	58	56.31	1.563	1.443		**0.241**		
6.Mankulam(10)	37	35.92	1.36	1.281		**0.155**		
With respect to all sub-populations	**93**	**90.29**	**1.9**	**1.58**	**0.331**	**0.206**	**0.321**	**0.357 (35.7%)**

C. vattayila

Three populations, Nelliamapathy, Kallar and Sholayar were screened for molecular assay.

ISSR markers

With respect to 9 ISSR markers used in the *C. vattayila* populations, a total of 46 fragments were scored of which, 19 (41.3%) in Nelliampathy, 18 (39.13%) in Kallar and 13 (28.3%) in Sholayar were polymorphic fragments. The band size generated by the nine primers ranged from 250 to 2000 base pairs. On an 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 which are low, while Sholayar has lowest gene diversity of 0.118.

When all the three populations, Nelliampathy, Kallar and Sholayar, were analysed together, total genetic diversity among populations (Ht) was 0.238 while within population diversity (Hs) was found to be very low as 0.147. The percentage of total genetic differentiation (38.3%) among the three populations (Gst= 0.383) was relatively very much higher than the mean *Gst* value (0.129) reported in many other species. This result indicated that 61.7% of the genetic diversity resided within the populations.

RAPD markers

With respect to 20 RAPD markers used, a total of 120 fragments were scored of which, 50 (41.7%) in Nelliamapthy, 64 (53.3%) in Kallar and 39 (32.5%) were polymorphic fragments. The band size generated by the nine primers ranged from 250 to 2500 base pairs. 1.27 out of an average 1.42 observed alleles in Nellaimapthy, 1.38 out of 1.53 observed alleles in Kallar and 1.26 out of 1.33 observed alleles in Sholayar were effective alleles.

The gene diversity was found to be 0.157 in Nelliampathy which is low while Sholayar was the least diverse with 0.14 value. The gene diversity in Kallar with a value of 0.213, was found to be moderate.

When all the three populations, Nelliampathy, Kallar and Sholayar, were taken together, the value of total genetic diversity among populations (Ht) was 0.331 while within population diversity (Hs) was found to be very low as 0.17. The percentage of total genetic differentiation (48.6%) among the three populations (Gst= 0.486) was relatively very much higher than the mean *Gst* value (0.129) reported in other species (Hamrick *et al*., 1992) which is more comparable with self pollinating species. This result indicated that only 51.4% of the genetic diversity resided within the populations. On analysis with ISSR and also with RAPD, within population diversity was found to be low in individual populations of *C. vattayila* than in the other three species screened in the present study (Table 5). An average of Nei's index, Hs = 0.147 (ISSR) and 0.17 (RAPD), at population level was lower than the mean value (H = 0.22) of many species (Nybom, 2004). Moreover, genetic differentiation and total gene diversity among populations were high, indicating the conservation of germplasm from all the populations. The results also indicate the possibility of inbreeding in *C.vattayila*.

The genetic distance between three populations is given in Table 6 which shows that Kallar is closer to Nelliampathy with a value of 0.0737 with respect to ISSR markers while Kallar is closer to Sholayar with a value of 0.182 on analysis with RAPD markers. The matrices of Nei's genetic distances were used to cluster the populations by the UPGMA method. The results, both through RAPD and ISSR marker analysis, show that Nelliampathy and Sholayar populations are far distant than Kallar though they are geographically closer (Figs. 4, 5). But when ISSR markers were used, Kallar is grouped with Nelliampathy and when RAPD markers were used Kallar is in the cluster with Sholayar. Galvan *et al.* (2003) suggested that ISSR would be a better tool than RAPD for phylogenetic studies. However, the

Dendrogram shows that Nelliampathy and Sholayar populations are far distant than Kallar though they are geographically closer.

Table 6. Genetic distance between populations of *C.vattayila*

Population	ISSR marker based		RAPD marker based	
	1	2	1	2
1. Nelliampathy	***		***	
2. Kallar	0.0737	***	0.4381	***
3. Sholayar	0.2184	0.2159	0.3989	0.1820

Fig. 4 Dendrogram for *C. vattayila* populations based on ISSR markers

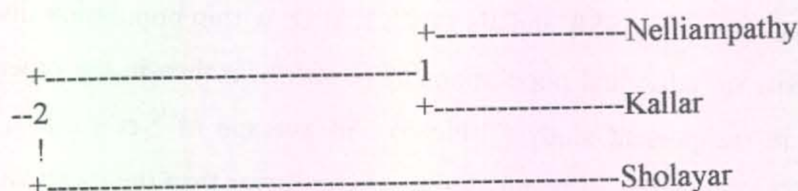
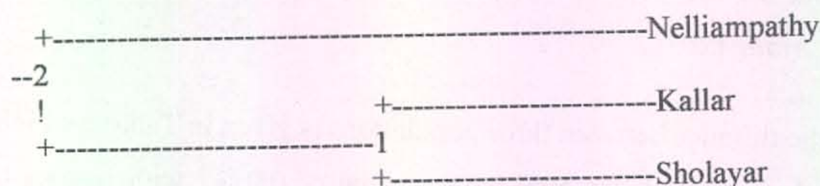


Fig. 5 Dendrogram for *C. vattayila* populations based on RAPD markers



C. travancoricus

ISSR markers

With respect to 9 ISSR markers used in the *C. travancoricus* populations, a total of 50 fragments were scored out of which 28 (56%) were polymorphic in Kottiyur population, 19 (38%) in Sholayar, 22 (44%) in Shendurney, 26 (52%) in Achenkovil, 30 (60%) in Arienkavu and 16 (32%) in Mankulam. The band size generated by the nine primers ranged from 250 to 2250 base pairs.

On an average, effective alleles were 1.37 out of 1.56 observed alleles in Kottiyur, 1.29 out of 1.38 observed alleles in Sholayar, 1.39 among 1.44 observed alleles in Shendurney, 1.39 out of 1.52 observed alleles in Achenkovil, 1.43 out of an average 1.6 observed alleles in Arienkavu and 1.27 out of 1.32 observed alleles in Mankulam.

The gene diversity was found to be 0.238 in Arienkavu which is moderately higher. It was 0.219 in Achenkovil, 0.218 in Kottiyur and 0.204 in Shendurney which are moderate values. Low gene diversity was seen in Sholayar (0.161) and Mankulam (0.145).

When all the six populations, were compared together, the value of total genetic diversity among populations (Ht) was 0.281 while within population diversity (Hs) was found to be very low as 0.197. The percentage of total genetic differentiation (29.8%) among the six populations (Gst= 0.298) was relatively much higher than the mean *Gst* value (0.129) reported in many species (Hamrick *et al.*, 1992). This result indicated that 70.2% of the genetic diversity resided within the populations.

RAPD markers

With respect to 20 RAPD markers, a total of 103 fragments were scored with respect to all the primers in *C. travancoricus*, of which 70 (67.96%) were polymorphic fragments in Kottiyur, 47 (45.63%) were polymorphic in Sholayar, 34 (33.01%) were polymorphic in Shendurney, 60 (58.25%) in Achenkovil, 58 (56.31%) in Arienkavu and 37 (35.92%) were polymorphic in Mankulam. The band size generated by the nine primers ranged from 250 to 2250 base pairs.

With respect to effective alleles, on an average 1.47 were seen out of 1.68 observed alleles in Kottiyur, 1.36 effective alleles out of 1.46 observed alleles in Sholayar, 1.26 out of 1.33 observed alleles in Shendurney, 1.43 out of 1.58 observed alleles in Achenkovil, 1.44 out of 1.56 observed alleles in Arienkavu and 1.28 effective alleles out of 1.36 observed alleles in Mankulam.

The gene diversity was found to be moderately high in Kottiyur (0.266), Achenkovil (0.241) and Arienkavu (0.241). But it was low in Sholayar (0.194), Shendurney (0.142) and Mankulam (0.155). When all the six populations, Kottiyur, Sholayar, Shendurney, Achenkovil, Arienkavu and Mankulam, were compared together, the value for total genetic diversity among populations (Ht) was 0.321 while within population diversity (Hs) was found to be moderate as 0.206. The percentage of total genetic differentiation (35.7%) among the three populations (Gst= 0.357) is relatively very much higher than the mean *Gst* value (0.129) reported in many other species. This result indicated that only 64.3% of the genetic diversity resided within the populations.

When ISSR and RAPD markers were compared, it was found that Sholayar and Mankulam are the two sites where low gene diversity was seen. Arienkavu, Achenkovil and Kottiyur are populations with moderately higher gene diversity with respect to both the markers. Shendurney was having moderate gene diversity with ISSR while it had low value on analysis through RAPD. Within the population genetic diversity was 70.2% for ISSR markers while it was 64.3% with respect to RAPD markers.

A higher level of genetic diversity in *C. travancoricus* (P = 86%, H = 0.285 with ISSR markers and P= 90%, H= 0.331 with RAPD) was detected in this study. The low genetic diversity of *C. travancoricus* at population level at Mankulam, Sholayar and Shendurney might be due to the restricted gene flow, gene drift, breeding with relatives as well as natural selection.

The genetic distances between populations are given in Table 7 (ISSR markers) and Table 8 (RAPD markers) which show that the closest populations are Achenkovil and Arienkavu. When the populations were grouped through UPGMA with ISSR data, populations from North and Central Kerala (Kottiyur, Mankulam and Sholayar) formed a group, while populations from South Kerala (Shendurney, Arienkavu and Achenkovil) formed another cluster (Fig.6). Hence, the dendrogram generated by the

ISSR matrix agrees better with the geographic origins of the genotypes than the dendrogram generated by the RAPD results. When RAPD data were analysed, there are three clusters (Fig.7), one comprising Achenkovil and Arienkavu, the second one with Sholayar and Mankulam and third one comprised of Kottiyur and Shendurney without any geographical relationship.

Table 7. Genetic distance between populations of *C. travancoricus* (ISSR)

Population	**1**	**2**	**3**	**4**	**5**
1. Kottiyur	***				
2. Sholayar	0.1333	***			
3. Shendurney	0.0922	0.1008	***		
4. Achenkovil	0.1593	0.1272	0.0636	***	
5. Arienkavu	0.2168	0.1850	0.0770	0.0325	***
6. Mankulam	0.0947	0.1246	0.1023	0.1261	0.1944

Table 8. Genetic distance between populations of *C . travancoricus* (RAPD)

Population	**1**	**2**	**3**	**4**	**5**
1. Kottiyur	***				
2. Sholayar	0.1898	***			
3. Shendurney	0.1755	0.1917	***		
4. Achenkovil	0.1839	0.1523	0.1729	***	
5. Arienkavu	0.1901	0.1784	0.1812	0.0625	***
6. Mankulam	0.2097	0.1562	0.1816	0.2054	0.2166

Fig. 6. Dendrogram for *C. travancoricus* populations based on ISSR markers

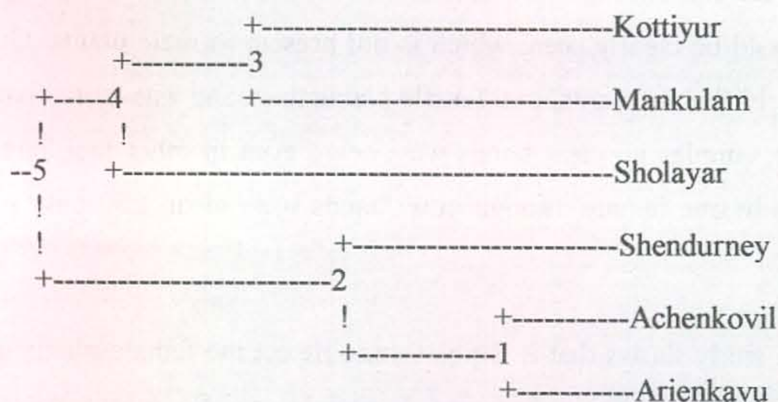
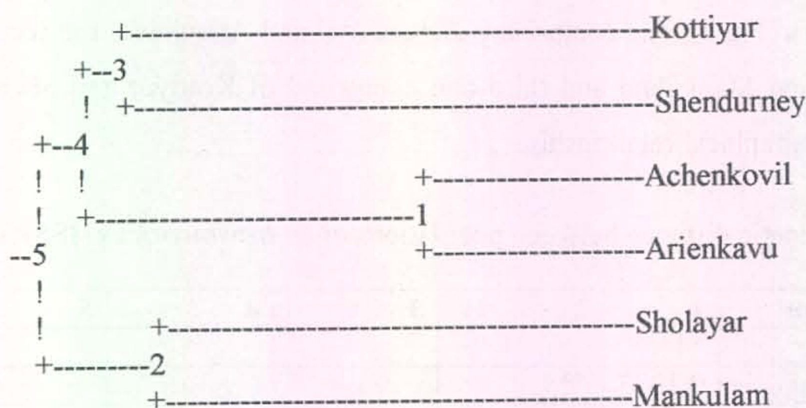


Fig. 7. Dendrogram for *C. travancoricus* populations based on RAPD markers



II. Sex determination

Ten male and ten female plants each of *C. brandisii* and *C. travancoricus* were used to develop a male specific or female specific marker for detection of female or male plants at seedling stage as mentioned earlier. Twenty RAPD and nine ISSR primers, which showed good polymorphism, were used for the analysis.

Though most of the primers resulted in good polymorphism, a male specific or a female specific band was not prominent in both the species for many of the markers used, except RAPD primer OPAU 02 in *C. travancoricus* and OPAW 20 in *C. brandisii*.

With regard to the RAPD marker OPAU 02, in *C. travancoricus*, the banding pattern was quite different for male and female. In female plants additional band with 250 base pairs could be clearly seen, which is not present in male plants. Out of the total 10 male and 10 female plants, in 6 female plants this band was quite visible (Fig. 8). In two female samples no clear bands were seen, even in other loci, due to poor amplification and in one female, though other bands were clear, 250 base pair band was not seen.

The present study shows that it is possible to detect the female plants using the RAPD marker OPAU 02. On refinement, a female-specific scar marker can be

developed from the above, for which further studies are to be carried out as was done in *Calamus simplicifolius*. A male-specific SCAR marker (CsMale1) with 509 base pairs in size was developed from RAPD markers in the dioecious rattan species *C. simplicifolius* (Mei Li *et al*., 2010).

Similar studies were conducted in other *Calamus* species which show that a putative female-specific marker was identified with an ISSR marker (ISSR 4) in *Calamus tenuis* (Sarmah and Sarma, 2010) where the marker produced a unique 600-bp fragment in female bulk DNA, which was absent in male bulk DNA.

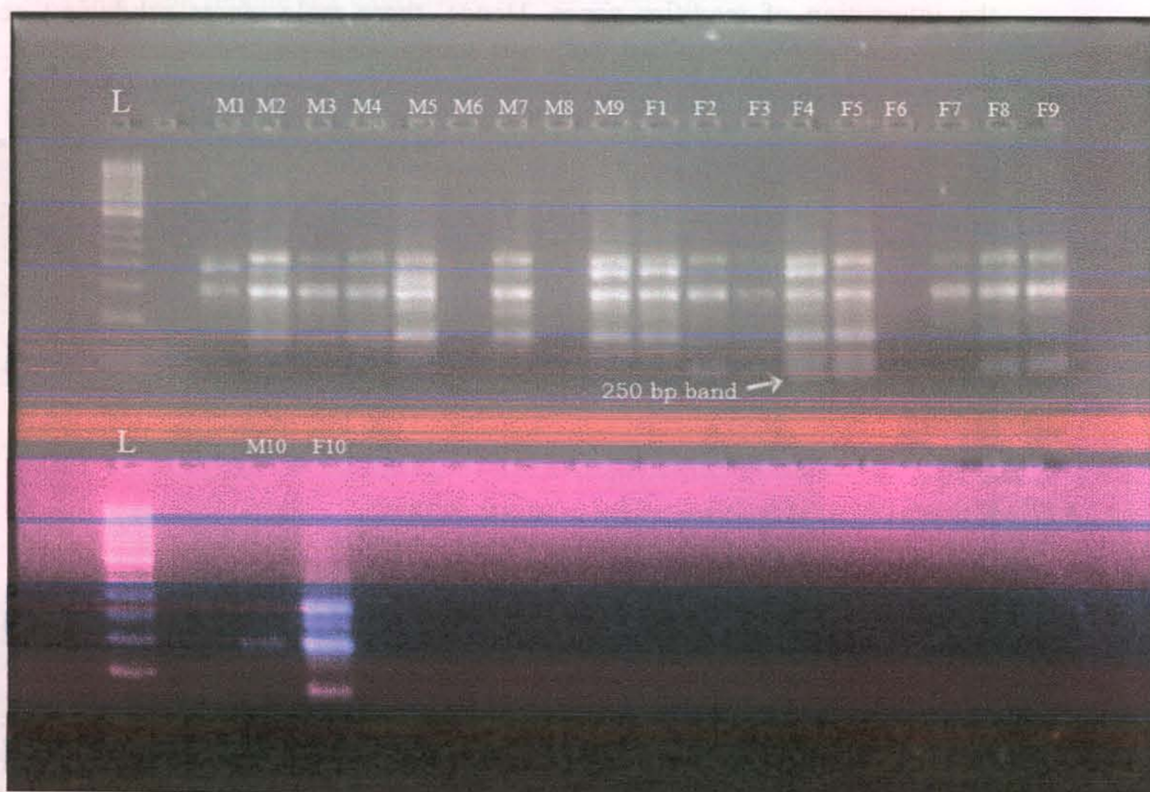


Fig. 8. Female specific 250 base pair band (OPAU 02) in *C. travancoricus*

With respect to the RAPD marker OPAW 20 in *C. brandisii*, the banding pattern was different for male and female. In male plants additional band with 750 base pairs could be clearly seen in 5 out of 10 males (Fig. 9), which is not present in the female plants except one (F4). OPAW 20 is a promising marker to detect male

plants in *C. brandisii.* In this species also a male-specific SCAR marker can be developed after further processing.

In many dioecious plants, males are 'inconstant', producing occasional fruits (Lloyd, 1975; Lloyd and Bawa, 1984). Self fertilisation of such plants in several species has provided genetic evidence that some of the males are heterozygous producing occasional fruits which have been identified as female plants like F4. In many dioeceous species, the male genotypes have a dominant suppressor of femaleness (Su^F)(Charlesworth, 2002). In few occasions, these males in heterozygous condition produce hermaphrodite flowers also and thereby showing androdioecy with the interaction of modifier genes. Hence, these male plants producing occasional fruits might have been suspected as female plants.

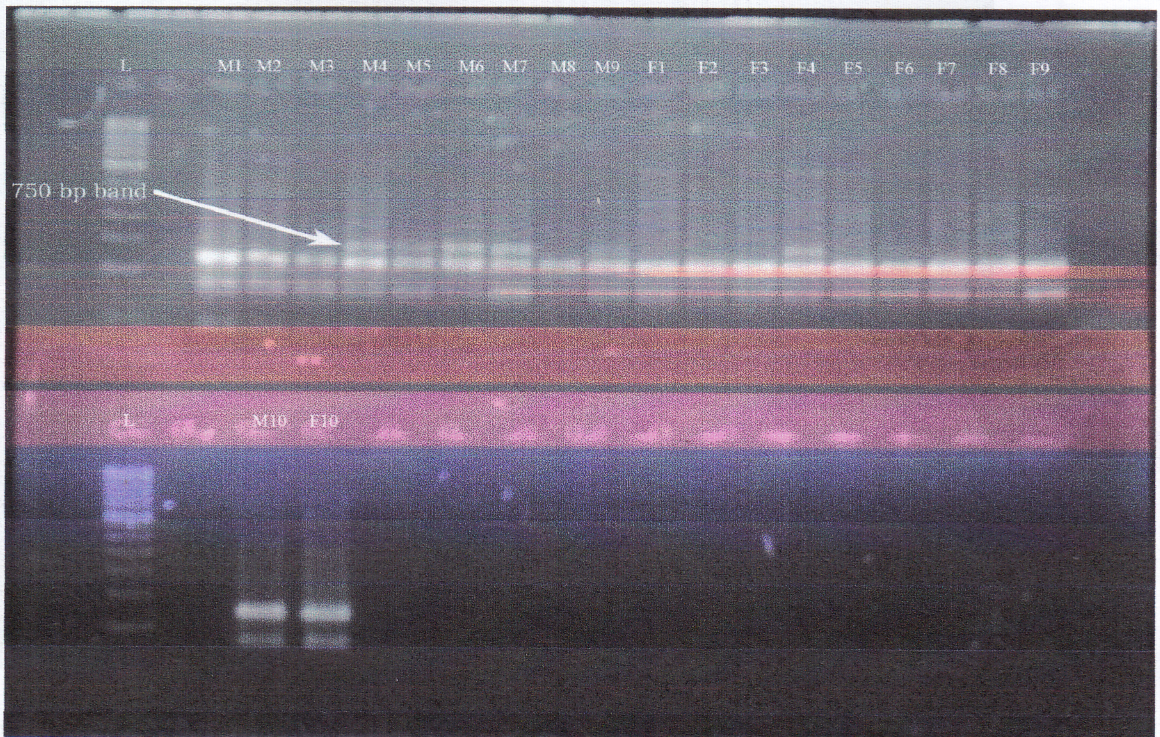


Fig. 9. Male specific 750 base pair band (OPAW 20) in *C. brandisii*

In the present study, the absence of female specific band in one female in *C. travancoricus* as well as the presence of male specific band in one of the females in *C. brandisii* was not surprising as andromonoecy (occurrence of separate male and

hermaphroditic flowers on the same individual) was reported in rattan species like *Calamus subinermis* (Lee, 1995).

Calamus species have been documented to be dioecious (Uhl and Dransfield, 1987). The occurrence of hermaphrodite plants in *Calamus* species indicates that the present classification of *Calamus* species needs to be revised (Chia, 2000). Chia (2000) also reported that the ratio of male, female and hermaphrodite plants in *Calamus subinermis* was different for different sites though females are more in numbers and the sex expression of rattan is affected by site and provenance. It is also noted that high female sex expression is seen in the loamy fertile soil than the coastal sandy soil. Lee (1995) also opined that favourable site conditions could enhance female sex expression. Lability of sex expression was reported in many dioeceous plants which are able to alter their sexual state in response to changes in the ambient environment and /or changes in size or age (Freeman *et al.*, 1980). In cucumber, sex expression is affected by hormone application, and endogenous hormone levels may be implicated in the process (Rafael Perl-Treves *et al.*, 1998).

As suggested by Chia (2000), in all the *Calamus* species in Kerala has to be screened thoroughly for the presence of hermaphrodite plants or occurrence of andromonoecy, gynomonoecy or any other mechanism.

III. & IV. Establishment of seed stands and reintroduction of the species to other suitable locations

The fruit set in *C. dransfieldii* was extremely low in its natural site at Dhoni and hence, seeds could not be collected as noted earlier. Among the other three species, *C. travancoricus*, *C. brandisii* and *C. vattayila*, high germination percentage were noticed in *C.vattayila* (85%) followed by *C. travancoricus* (70%). The seeds of *C.vattayila* started germination after 30 days of sowing and continued till 55 days. The period of maximum seed germination in *C. travancoricus* was 25 - 48 days after sowing. The germination percentage of *C. brandisii* seeds was very low (30%) and the period of germination was 40-50 days after sowing.

Observations recorded from the field show that overall percentage of survival rate in Malayattur forests for *C. vattayila* and *C. travancoricus* are 64% and 76% respectively (Table 8). Many of the seedlings were damaged by wild pigs which churn the forest soils and destroy the seedlings.

Table 8. The survival % in the seed stands at Malayattur Forest division.

Species	Populations	No. of seedlings planted in Malayattur	No. of seedlings survived	Percentage of survival
C. vattayila	Nelliampathy	150	118	78%
	Kallar	200	107	53.5%
	Sholayar	150	98	65%
C. travancoricus	Kottiyur	30	18	60%
	Sholayar	50	38	76%
	Pandimotta	150	123	82%
	Achenkovil	100	79	79%
	Arienkavu	100	76	76%
	Mankulam	70	44	63%

Seedlings of *C. brandisii, C. vattayila* and *C. travancoricus* were planted in the KFRI arboretum. Moreover, representative samples of each species from different populations were also added to the existing collection at KFRI rattan germplasm.

4. SUMMARY AND CONCLUSIONS

The present study carried out by employing 20 RAPD markers and 9 ISSR markers, analysed the gene diversity and related parameters, within and among 12 populations of four endemic and endangered species, i.e. *Calamus brandisii, C.dransfieldii, C. travancoricus* and *C. vattayila* growing in the Western Ghats. The results showed high gene diversity in the lone population of *C. dransfieldii* in Dhoni Forest of Olavakkode Division. With regard to the two *C. brandisii* populations screened, Shendurney is more diverse than Bonacaud and around 80 percent of the gene diversity resides with in populations. The populations are moderately diverse.

Within population gene diversities are low in three *C. vattayila* populations. But, intensity of species level diversity is good enough and genetic differentiation between these populations is very high in the range of 38 to 48 percent indicating the possibility of restricted gene flow or inbreeding. Cluster analysis showed that Nelliampathy and Sholayar populations are far distant (though they are geographically closer) than Kallar. Analysis of six populations in *C. travancoricus* shows that the gene diversity in Kottiyur, Arienkavu and Achenkovil are moderately high while they are low in Shendurney, Sholayar and Mankulam. In this species also, genetic differentiation between populations is high as 30 to 36 percent indicating the likelihood of limited gene flow or inbreeding. Dendrogram drawn from the data showed a closer relation between Achenkovil and Arienkavu and between Sholayar and Mankulam.

Rattans are reported to be dioecious and to determine the sex of the plants at the seedling stage, studies using 20 RAPD and 9 ISSR markers were conducted which showed that the RAPD marker, OPAU 02, is effective to detect female plants in *C. travancoricus* and the RAPD marker OPAW 20 for detecting male plants in *C. brandisii.* The study also indicated the possibility of hermaphrodite flowers/ plants in *Calamus* sp. which should be confirmed through further field studies.

As envisaged, efforts were made to establish seed stands for which thousand seedlings of *C. vattayila* and *C. travancoricus*, originated from nine populations from

Kerala, were planted in Kodanad Forest Range of Malayattur Division. Seedlings originated from different populations of all the four species were raised in the nursery and a total of 1500 seedlings of *C. vattayila* and 500 seedlings of *C. travancoricus* were handed over to the Research Range Office, Kodanad for enriching the existing populations. Few seedlings were also planted in the KFRI arboretum and Palmatum. On summing up the present investigations, the following conclusions are drawn.

1. The one and only population of *C. dransfieldii* displays high gene diversity. This population has extremely low fruit set and natural regeneration.
2. *C. brandisii* populations are moderately diverse and 80 percent of the gene diversity resides with in populations. Population at Shendurney is more diverse than Bonacaud.
3. Intensity of species level diversity is good enough in *C. vattayila* while within population gene diversities are low. Genetic differentiation between populations is very high to the tune of 38 to 48 percent indicating the possibility of restricted gene flow or inbreeding.
4. In *C. travancoricus* gene diversity in Kottiyur, Arienkavu and Achenkovil are moderately high while they are low in Shendurney, Sholayar and Mankulam. Genetic differentiation between populations is high as 30 to 36 percent indicating the likelihood of limited gene flow or inbreeding.
5. By using the RAPD marker OPAU 02 it is possible to detect female plants in *C. travancoricus*. Like wise, OPAW 20 is a marker for detecting male plants in *C. brandisii*. The study also shows the possibility of hermaphrodite flowers/ plants in *Calamus* sp. and hence, classification of rattans under dioecious nature needs to be verified and revised if needed.
6. Seed stands established at Kodanad Range which encompass seedlings originated from nine populations of *C. vattayila* and *C. travancoricus* is expected to produce enough seeds for raising plantations.
7. Seedlings of the selected endemic and endangered species planted in the existing populations would lead to genetically diverse and robust populations while those planted in the KFRI arboretum would build up the *ex situ* conservation plots.

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