Genetic diversity of Indian *Cycas* species assessed using RAPD markers

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Abstract

RAPD analysis was carried out to determine the genetic variability of ten species of Indian *Cycas.* Randomly collected leaf materials were used in the present study is to assess the extent of Random amplified polymorphic DNA (RAPD) variation to discriminate and construct the genetic relationship between ten species of Indian *Cycas*. Twenty oligonucleotide decamer primers were screened and six primers were selected to analyze the polymorphism in *Cycas* species. The relative similarity between species was estimated by Jaccard's similarity index and clustered in UPGMA, is generally in accordance with taxonomic position. Similarity index between the species ranged from 0.11 to 0.62 with 98.1% polymorphism. RAPD analysis was efficient to address the genetic diversity of *Cycas* species and contributed to understand the adaptation to the taxonomic implications.

Keywords: *Cycas,* RAPD, genetic diversity, Polymorphism

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Introduction

The widespread loss of the World's biological wealth is the most serious global crises today. Regarding the flora, systematic collection and preservation of as many species as possible, particularly the endangered plant species is the most effective means of preventing the crisis. The Order Cycadales is represented by ten genera and about 343 species from the tropical

and sub-tropical parts of the world (Calonje et al., 2015). Asian cycads are represented by single genus *Cycas* with 105 species and subspecies (Donaldson, 2003). Ten species (*C. annaikalensis* Singh and Radha, *C. beddomei* Dyer, *C. circinalis* L., *C. nayagarhensis* Singh and Radha, *C. orixensis* (Haines) Singh and Khuraijam, *C. pectinata* Ham., *C. rumphii* Miq, *C. sphaerica* Roxb, *C. swamyi* Singh and Radha and *C. zeylanica* (J.Schust.) A.Lindst. and K.D.Hill,) have been reported so far from India. These plants grow in the wild habitats occur in northeast (Odhisa, Bihar, Sikkim, Bhutan, Assam) and in southern states (Andhra Pradesh, Tamil Nadu, Kerala, Karnataka) of India and also reported from Andaman Islands (Singh and Radha, 2006; Lindstorm and Hill, 2007; Singh et al., 2015).

Cycads have probably been used by people since prehistoric times and they have been traded for food, basketwork, medicine, magic, ceremonies, decoration, and many other purposes. In India, *Cycas* leaves are used as food, medicine, garden plant, ornamentation as well as used in cultural and ethical functions. The male cone of *Cycas* is used as medicine and insect repellant; the starchy endosperm is used in many cuisines prepared by the natives of Kerala, Odhisa, Assam and Andaman and Nicobars islands. (Thieret 1958; Whiting 1963; Radha and Singh, 2008).

The taxonomy of *Cycas* at the species level has always portrayed a great difficulty. Pant (1973) while citing opinions on the actual numbers of species of *Cycas* cautioned that " the total number of specific names in the literature is much larger and the specific classification of the genus is at present in a confused state". Therefore, until each of the species of *Cycas* is revised on the basis of detailed studies in their morphological, genetic and other characteristics and the range of variation, it would perhaps be better to recognize all the generally accepted species as distinct"-.

In the case of *Cycas* taxonomy, species are delimited on the basis of characteristics of the reproductive organs and leaves. All Indian species of *Cycas* except *C. beddomei* are enunciated as DD (Data deficient) categories in the IUCN report (Donaldson, 2003). *C.beddomei* has been enunciated under Critically Endangered Plant (CR). The conservation status of the species is due to anthropogenic activities, such as loss and alteration of habitat, introduction of invasive species, manmade forest fire etc. Except *C. beddomei*, all the Indian *Cycas* species externally appear identical until the reproductive organs flourish. Currently there are five species of morphologically similar *Cycas* found in the Western Ghats and Eastern Ghats. The complex genetic structure sometimes cannot be fully assessed with morphological and anatomical differentiation; the use of molecular markers, such as RAPD,

RFLP, AFLP, ISSR and intergenic spacers of organelle DNAs provide tools to assess genetic variation between and within populations to understand such complexities (Culley and Wolfe, 2001; Decastro et al. 2006; Jian et al. 2006; Mekanawakul et al. 2003; Radha and Singh, 2011).

Genetic diversity in natural populations is of great concern because the amount and distribution of genetic diversity is likely to affect the evolutionary potential of species and populations (Futuyma, 1998). Thus, genetic diversity is not only essential for the study of the endangered flora, but also to elaborate strategies of conservation and rational use of genetic resources. Development of marker study would be highly useful in taxonomy, evolutionary and phylogenetic study of the plants and acclimatizing in the natural habitats. RAPD marker studies have been encouraged to reveal the genetic polymorphism in several plant genera due to technical simplicity and rapid results. This method has already been applied in the systematic study of Thailand *Cycas* species (Sangin et al. 2006) and sex determination in *Encephlartos natalensis* (Prakash and Staden 2006). Henceforth, the present study was aimed to assess the competence of RAPD markers in investigating genetic relationship of Indian *Cycas*, which is important for germplasm conservation and species identification of these rare, old, commercially and medicinally important plants.

Materials and Methods

Plant Materials

The plant materials for the present study were collected from different habitats of *Cycas* (Table 1). Pinnae (leaflets) of both fresh as well as dried samples were used for genomic DNA isolation. Collected materials were given accession number and stored at – 70^0C until use for DNA isolation. The genomic DNA was extracted by CTAB method. (Doyle and Doyle, 1990).

RAPD PCR amplification

Genomic DNA polymorphism was determined by the Random Amplified Polymorphic DNA (RAPD) method (Williams et al., 1990). Amplification reactions were carried out in a volume of 25 µl containing 50-100 µg of genomic DNA, 1X buffer (100 mM Tris (pH 9.0), 500 mM KCl, 0.1% Gelatin), 0.2 mM dNTPs, 2-3 mM $MgCl_2$, 2.25 U of *Taq* DNA polymerase and 0.2 µM of primer. All analyses reagents used are from Bangalore Genei, Random decamer

primer, Series B OPB1-OPB20 (Operon Tech., Alameda, USA) was used for the present study. DNA amplification was conducted in Applied Biosystem (2720) thermal cycler. PCR setup was programmed with the following temperature profile. One cycle of 5 min at 94^0 C followed by 45 cycles of 1 min at 94^0 C,1 min at 35^0 C and 2 min at 72^0C. Final extension at 5 min at 72^0C and hold at 4^0C. PCR products were subjected to 1.5% agarose gel electrophoresis run at 60-90V and DNA banding were visualized by ethidium bromide staining. The size of amplified products was determined with Gene Rular DNA Ladder Mix (Fermentas).

Data Analysis

Only the primers which displayed reproducible, scorable and clear bands were taken for the data analysis (Table 2). The amplified fragments (bands) generated using 6 oligonucleotide primers were scored manually for their presence (denoted as '1') and absence (denoted as '0'). The statistical analysis was performed using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) software (version 2.02i, Rohlf, 1998). The genetic similarity (GS) values between pairs of samples were estimated according to Jaccard's similarity coefficient (Gs_{ij}= a/a+b+c), where Gs_{ij} is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The values of SM (Similarity Index) which was used to develop a dendrogram using the UPGMA (Unweighted Pair Group Method of Arithmetic Averages; Sneath & Sokal, 1973) and SAHN (Sequential Agglomerative Hiearchial Non-overlapping) clustering algorithm in order to cluster the samples.

Results

RAPD polymorphism

Random Amplified Polymorphic DNA (RAPD) markers were used to assess genetic diversity among the Indian cycads. Nine species and one variety of *Cycas* have been collected from different wild habitat and gardens for the present study (Table 1).

In the analysis twenty oligonucleotide decamer primers were screened (OPB1-OPB20) for the genomic DNA amplification and six primers (OPB4, OPB5, OPB7, OPB8, OPB13, OPB19) were selected to amplify DNA. Among the six primers, five primers generated

polymorphic bands where primer OPB5 generated monomorphic bands The DNA fragments that were amplified by selected primers were scored as present '1' or absence '0' for all studied species. A total number of fifty three fragments generated with size ranging from 100-1200bp were scored (Figure 1). The similarity indices showed that the relationship between the ten species found in the range of 0.11 to 0.62 (Table 3). The dendrogram based on UPGMA analysis cluster analysis of the 53 bands showed two clusters include five clades of studied Indian cycad. Maximum similarity was found between *C.circinalis* and *C.annaikalensis* (62%) and the least similarity was found in between *C.swamyi* and *C. orixensis*(11%). The dendrogram (Figure 2) classified the ten taxa into five clades. Clade I consisted of the *C.annaikalensis, C.circinalis* and *C.swamyi*; Clade II includes *C.beddomei* and *C.species*; Clade III consists of *C.pectinata*; *C.rumphii* and *C.zeylanica* are clustered together in Clade IV and Clade V consists of *C. sphaerica* and *C. orixensis.*

Table 1 Details of *Cycas* species used in present study

S.NO	Name of the species	Accession code	Place/state
1.	*C. annaikalensis*	Ca	Palaghat/Kerala
2.	*C. beddomei*	Cb	Tirupathi/A.P
3.	*C. orixensis*	Cco	Odhisa, 2011*
4.	*C. circinalis*	Cc	Malapuram/Kerala
5.	*C. pectinata*	Cp	Manipur, October 2010*
6.	*C. rumphii*	Cr	Andaman, India
7.	*C. species*	Cs	Mamandur, AP.
8.	*C. sphaerica*	Csp	Srikakulam/A.P
9.	*C. swamyi*	Csw	Nagamangla, Karnataka, 2010*
10.	*C. zeylanica*	Cz	GGSIPU Garden, 2011*

*samples collected from garden with known origin

Discussion and Conclusion

Cycas occur naturally in all three well-marked physiographic regions of India. Among the studies plants, five species are (*C.annaikalensis, C.circinalis, C.sphaerica, C.orixensis* and *C. species*) similar in external appearance, but close observation on morpho-anatomy of both vegetative and reproductive organs utilized in the systematic studies. For example, *C. swamyi, C. circinalis* and C. *annaikalensis* are found in the same geographical region i.e., Western Ghats but all these species grow in different habitats that might have resulted in

their variations and speciation along with other factors like reproductive isolation caused by genetic drift (Radha and Singh 2011). The similarity in between *C.circinalis* / *C.annaikalensis* (0.62) and *C.circinalis* /*C.swamyi* (0.36) and absence of mucilage canals, dimension of the leaflet, frequently occur isotomous dichotomous branched stems distinguish *C.swamyi* from the other two Western Ghats *Cycas* species (Singh and Radha 2008). *C. beddomei, C.species, C. sphaerica* and *C. orixensis* are reported from the Eastern Ghats. *C. beddomei* is a palaeoendemic species and its distribution is limited to the South Eastern Ghats i.e., Cuddapah hills which constituent of dry deciduous, scrubby and open sunny forests. *C. beddomei* is morphologically distinct from all other Indian species in their external appearance. *C.species* is growing in the same region which is totally distinct from *C.beddomei*. The systematic position of *C.species* is under investigation. However, it has been added with *C.sphaerica* by Lindstorm and Hill (Lindstorm and Hill, 2007). The similarity in between *C. sphaerica* and *C.species* is very least (0.36) and they grouped separately (Figure 1) *C.sphaerica* and *C. orixensis* are found in the north Eastern Ghats. The distribution and pattern of the vegetation and endemic plants in the Eastern Ghats is quite different from the Western Ghats. The species rich zones are isolated primarily because the Eastern Ghats don't form continuous range instead consists of a rather broken hill ranges with plains in between. Thus the isolation and restricted distribution of the narrow endemic plants are much more pronounced in Eastern Ghats due to the geographical, ecological, edaphic and climatic barriers in comparison to the Western Ghats.

Table 2 Primer employed and RAPD marker obtained from DNA amplification

Primer	**Sequence 5' - 3'**	**No of bands (range from 100-1200bp)**		
		Polymorphic	Monomorphic	Total
OPBB 4	GGACTGGAGT	9	0	9
OPBB 5	TGCGCCCTTC	10	1	11
OPBB 7	GGTGACGCAG	10	0	10
OPBB 8	GTCCACACGG	3	0	3
OPBB 13	TTCCCCCGCT	10	0	10
OPBB 19	ACCCCCGAAG	10	0	11
		52	1	53

Table 3 Similarity matrix of Indian *Cycas* species based on RAPD data

	Ca	Cc	Cb	Cco	Csp	Csw	Cp	Csph	Cr	Cz
Ca	1.00000									
Cc	0.62500	1.00000								
Cb	0.28571	0.35000	1.00000							
Cco	0.25000	0.33333	0.31250	1.00000						
Csp	0.31578	0.38888	0.36842	0.18750	1.00000					
Csw	0.36842	0.36842	0.22727	0.11111	0.19047	1.00000				
Cp	0.25000	0.25000	0.24000	0.26315	0.26086	0.15384	1.00000			
Csph	0.34782	0.47619	0.23076	0.19047	0.36363	0.29166	0.29629	1.00000		
Cr	0.21739	0.21739	0.26086	0.22222	0.28571	0.21739	0.23076	0.43478	1.00000	
Cz	0.34615	0.34615	0.33333	0.20833	0.41666	0.29629	0.34482	0.48148	0.60869	1.00000

C. pectinata grows wildly in Assam and Bihar on the east of the Indo-Gangetic alluvial plains. This species is robust and have characteristic pectinate megasporophylls unlike rest of the Eastern Ghats species. The morphological variations have been observed in the *C.pectinata* population found throughout Burma, Sikkim, Thailand and China and form a *C.pectinata* complex. C. *rumphii* and *C. zeylanica* occur on the islands of Andaman and Nicobar but reportedly they also occur in the coastal forests of Bangladesh (Radha and Singh 2011).

Most of the studies on cycads that deal with taxonomy, species identification are done based on morpho-anatomical characters of both vegetative and reproductive organs (Stevenson 1992, Radha 2007) and molecular markers like RAPD, RFLP (Sangin et al 2006), AFLP (Mekanawakul et al. 2007; Radha and Singh 2011) and Isoenzymes (Cully and Wolfe 2002; Xiao et al. 2004). Results of the RAPD and AFLP of different species of Thailand *Cycas* are grouped on the basis of their geographical distribution, where in the case of RFLP results are not confirmed with geographical distribution, they showed close conformity with morpho-anatomical characteristic features (Sangin, 2006). AFLP studies six Indian species of *Cycas* was grouped and correlated with geographical distribution of plants (Radha and Singh, 2011)

In the present study, similarity indices between the species ranged from 0.11 to 0.62 with 98.1% polymorphism. The least genetic similarity (0.11) was between *C. orixensis* and *C.swamyi* which geographically and are morpho-anatomically different. The greatest similarity was found in between *C. annaikalensis* and *C. circinalis* (0.62) and these species are growing in the same geographical region and differentiated on the basis of reproductive organs. Higher similarity indices suggest that the closer genetic relationship of the studied

species, while the lower similarity indices suggest that the species have farther genetic variation.

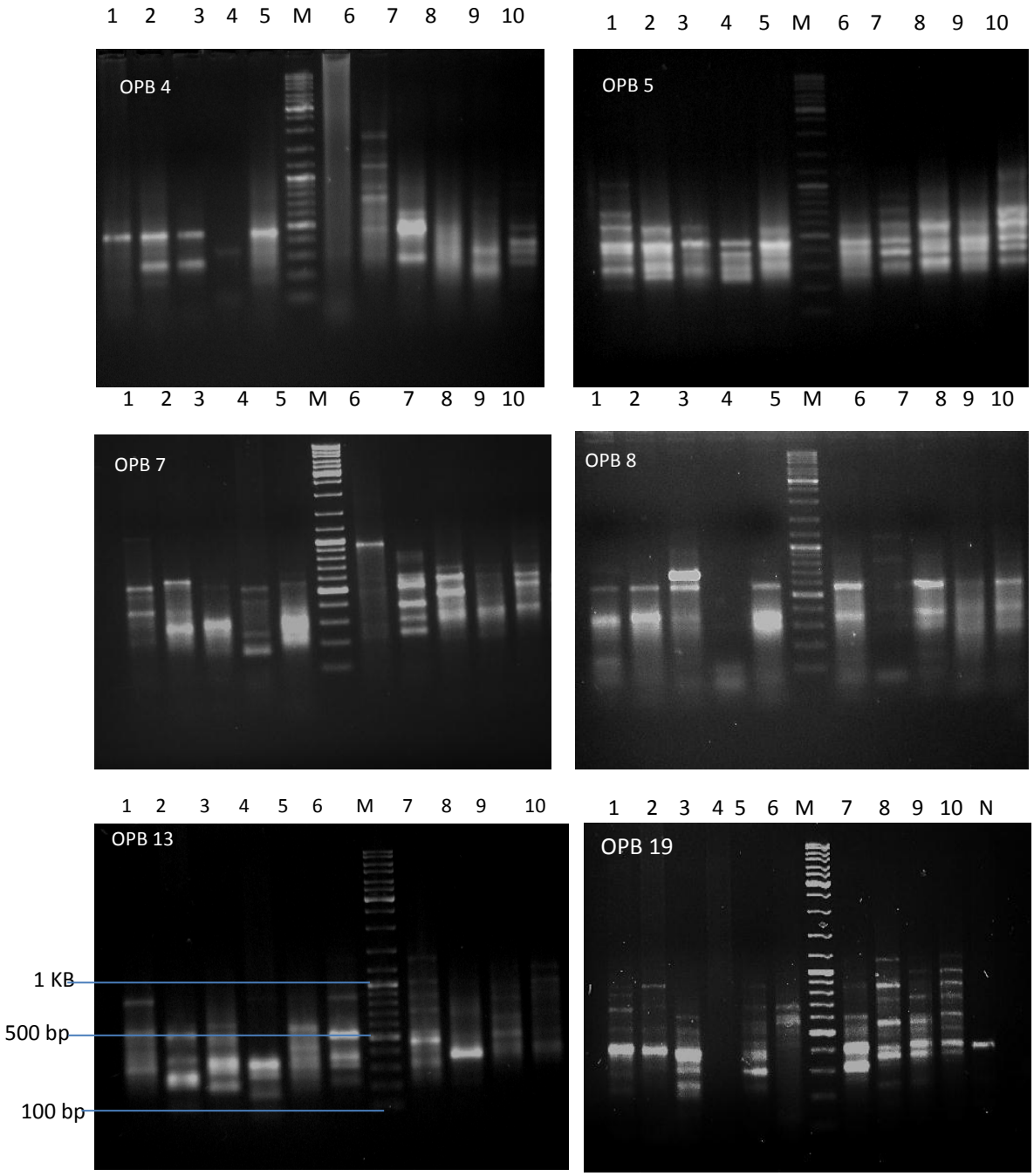

Figure 1. RAPD amplification of nine species and one variety of Indian cycads using the oligo nucleotide primers. M: Gene ladder mix.

1. *C.annaikalensis,* 2. *C.circinalis* 3. *C.beddomei* 4. *C. orixensis* 5. *C.species* 6. *C.swamyi* 7. *C.sphaerica* 8. *C.pectinata* 9. *C.rumphii* 10. *C.zeylanica.*

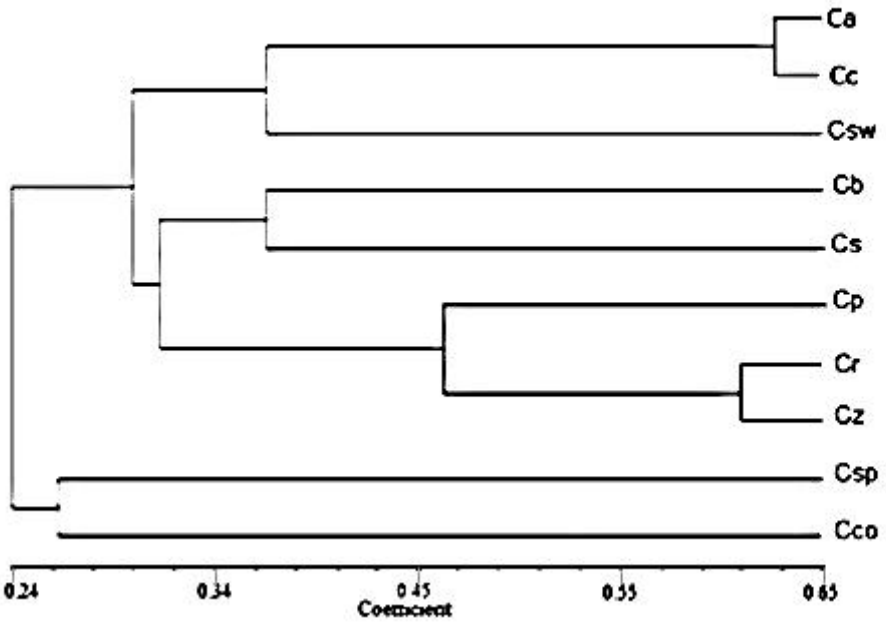

Figure 2. Dendrogram showing clustering between *Cycas* species generated from Similarity Index based on UPGMA.

The grouping of *C.species/ C.beddomei* and *C.sphaerica* / *C. orixensis* with 0.36 and 0.19 similarity indices clearly supported the distinguishing of the species from same geographical region. However, *C.zeylanica* and *C.rumphii* with 0.60 shows their identical origin and morpho-anatomical features include broad leaflets and spongy layer in the seed coat. Dendrogram of RAPD seems to show a correlation with geographical distribution of the studied plants and also shows their close affinity of morpho-anatomical characters too. Thus we can conclude that RAPD analysis was highly efficient in addressing genetic diversity that contributing to an understanding of adaptation of environment, conservation and taxonomic implication of this highly endangered and rare plant group.

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