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Full Length Research Paper

Amplified fragment length polymorphism (AFLP) studies on Indian *Cycas* species

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Amplified fragment length polymorphism (AFLP) technology was used to reveal the genetic variation in six species of *Cycas* collected from eleven natural populations. Two sets of primer with 4-selective nucleotides were used in this study and 78% polymorphism was found. The results correlated with geographical distribution of the plants and grouped into four. This study is highly useful in the delimitation of Indian *Cycas* species which has been enunciated as highly endangered taxa by IUCN Red Data List.

Key words: Cycads, *Cycas*, amplified fragment length polymorphism (AFLP), polymorphism.

INTRODUCTION

Cycads are ancient plants with a long continuous line of hereditary and these are the oldest group of living seed plants appearing back from the Pennsylvanian era, that is, approximately 300 million years ago. Cycads dominated the Mesozoic forest along with the other gymnosperms. The Family Cycadaceae consists of single genus *Cycas* and about 110 species. These plants are distributed in the tropical and sub-tropical region of the world. Eight species and one variety of *Cycas* have been reported from India (*Cycas annaikalensis* Singh and Radha, *Cycas beddomei* Dyer, *Cycas circinalis* L., *Cycas pectinata* Ham., *Cycas sphaerica* Roxb, *Cycas rumphii* Miq and *Cycas zeylanica* (J.Schust.) A.Lindst. and K.D.Hill, *Cycas swamyi* Singh and Radha and *Cycas circinalis* var. *orixensis* Haines). Field studies have shown that the majority of cycads populations in the wild are either threatened, critically endangered or on the brink of extinction and also have been listed in the threatened categories of IUCN. This is because of their narrow distribution, small population sizes, various threats to populations and their habitats (Osborne, 1995; Donaldson, 2003). Most of the Indian *Cycas* species, except *C. beddomei* are morphologically similar and are difficult to distinguish in the vegetative state. Although, the species of cycad can be generally identified from coralloid roots, secondary metabolites and from isozyme profiles, these physiological and morpho-anatomical properties are also affected by the growth conditions and the environment making the closely related species difficult to identify. Understanding of the genetic variation within and between populations is therefore, essential for the establishment of effective and efficient conservation plans (Hamrick and Godt, 1990).

Amplified fragment length polymorphism (AFLP) is an ideal marker system for resolving diversity among individuals, populations and species (Mueller and Wolfenbarger, 1999). These markers have been utilized by Mekanawakul et al. (2003) to study species diversity and relationships of Thailand *Cycas* species. To assess the relationships of various species of *Ceratozamia* in Mexico, De Castro et al. (2006) and Jian et al. (2006) applied this technique to investigate genetic variation within and among natural populations of *Cycas fairylakea* in China. In this study, AFLP markers have being applied for the first time to analyze the genetic variation of Indian *Cycas* species.

MATERIALS AND METHODS

Plant materials

The plant materials for this study were collected from different habitats of Indian *Cycas* (Table 1). Pinnae of both fresh as well as preserved samples were used as tissue for DNA isolation. The

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Table 1. Details of Indian *Cycas* species used in this study.

Name of the species	Accession code	Sampling location in India
C. annaikalensis	IPL 03, IPL 08 new	Kerala, India
C. beddomei	IPL O8 old , IPL 10	Andhra Pradesh, India
C. circinalis	IPL 02, IPL 09 new	Kerala, India
C. circinalis var.orixensis	IPL 04,06,13, 14	Orissa, India
C. swamyi	IPL 09 old	Karnataka, India
C. zeylanica	IPL 11	*GGSIPU, garden, Delhi

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pinnae were lyophilized for 24 h at -70°C using Virtis lyophiliser before DNA isolation.

DNA isolation

Molecular studies were done at The Energy Research Insititute, New Delhi. Tissue samples for investigating the *Cycas* species were collected from fresh and dried leaves.

CTAB method (Doyle and Doyle, 1990) was used to isolate total genomic DNA. 100 mg of lyophilized plant material was ground to fine powder in mortar and pestle using quartz sand. The powder was transferred immediately into 5 ml of pre-warmed (65°C) isolation buffer, incubated for 30 min at 65°C in water bath and mixed gently every 10 min. Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed. The slurry was centrifuged at 10,000 rpm at 24°C for 10 min. aqueous phase was transferred to fresh tubes. 20 µl of RNase A (10 mg/ml) was added and incubated for 30 min at 37°C. Again, equal volume of chloroform: isoamylalcohol was added, mixed gently and centrifuged at 10000 rpm at 24°C for 20 min. Upper aqueous phase was transferred to glass centrifuge tubes using large bore pipette. 0.6 volume of ice-cold isopropanol was added, and mixed gently but thoroughly by inverting the tubes several times. At this stage, the DNA-CTAB complex was precipitated out, and then centrifuged at 6000 rpm at 4°C for 10 min to pellet out the DNA. The pellet was washed with washing solution (70% ethanol containing 10 mM ammonium acetate) by gently agitating and collected by centrifugation (10 min, 5000 rpm, 4°C). The pellet was dried and 500 µl of M.Q. water was added to the DNA to dissolve overnight. All purified DNA was quantified on agarose gel by comparing with uncut lambda DNA of known concentration.

AFLP assay

The AFLP procedure was performed using protocols and kits of Life Technologies following principle developed by Zabeau and Vos (1993). Genomic DNA (300 ng) was digested simultaneously with *Eco*RI and *Mse*I (1.25 U/µl each) in a reaction volume of 25 µl in restriction buffer. *Eco*RI and *Mse*I adapters were subsequently ligated to digested DNA fragments.

Preamplification

The ligated products were subsequently pre-amplified using primers complementary to the adapter sequences and the restriction sites. The following cycling parameters were used for pre-amplification: 20 cycles of 90°C for 30 s, 56°C for 60 s and 72°C for 60 s. An aliquot of the preamplified product was checked on the gel and smears were observed. The amplified product was Primer labeling: Only the *Eco*RI primer was end-labeled using γ- ^{32}P ATP and T_4 polynucleotide kinase.

Selective amplification

A diluted aliquot of the pre-amplified mix was used as a template for selective amplification. The primers used are *Eco*RI +3 and *Mse*I +3 primers. The following cycle profile ensured optimal primer selectivity: 1 cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, this cycle was followed by 12 cycles in which the annealing temperature was lowered by 0.7°C per cycle. Following this, 23 more cycles were performed with the following profile: 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

Conventional and 4- select AFLP

The conventional AFLP protocol developed by Vos et al. (1995) was used with minor modifications. In brief, DNA (approximately 200 to 250 ng) was digested in 25 µl volume using 5 µl of 5X restriction ligation buffer containing ATP, 2.5 µl of 0.5 M NaCl, 1.25 µl of BSA (1 mg/ml), 2.5 unit of *Mse*I and 5 unit of *Eco*RI at 37°C for 2 h followed by enzyme inactivation at 70°C for 10 min. Ligation was done at 20°C for 2 h by adding 5 pMol of *Eco*RI adaptor, 50 pMole of *Mse*I adaptor and 1 unit of T4 DNA ligase to the digestion reaction. The ligation mix was diluted 1:10 times in TE buffer (10 mM Tris 0.1 mM EDTA) and 2 µl of diluted ligation reaction was used as template for amplification with adapter specific primers *Eco*RI +A and *Mse*I + C in a total of 20 µl volume. The PCR reaction was performed in a Gene Amp PCR 9700 thermal cycler using the following cycling parameters: 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The pre-amplified mix was diluted 50 fold for selective amplification. Selective amplification was done using *Eco*RI and *Mse*I primers with 3 selective nucleotides in a total of 10 µl reaction volume. The PCR parameters were: 1 cycle of 30 s at 94°C, 30 s at 65°C and 72°C for 60 s. The annealing temperature was reduced by 1°C per cycle during the first 11 cycles, and then 23 cycles were performed at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. The samples were size-fractionated on 6% polyacrylamide gel and the fragments were detected by autoradiography. For 4-select AFLP, the selective amplification was done using *Mse*I primers with 4 selective nucleotides. The primers were modified by adding one more selective nucleotide at the 3' end and deleting one nucleotide from the 5' end to keep the overall length of primer unchanged. These modified *Mse*I primers with 4 selective nucleotides (4-select) were used in conjunction with γ^{32}P-ATP labeled *Eco*RI primers with

Table 2. Sequence of the primers employed for the AFLP analysis.

Name	Enzyme	Type	Sequence (5'->3')
E-A	*Eco* RI	Adaptor (+)	GAC TGC GTA CCA ATT CA
M-C	*Mse* I	Adaptor (+)	GAT GAG TCC TGA GTA AC
E-AAC	*Eco* RI	Primer +3	GAC TGC GTA CCA ATT C AAC
E-ACA	*Eco* RI	Primer +3	GAC TGC GTA CCA ATT C ACA
E-ACC	*Eco* RI	Primer +3	GAC TGC GTA CCA ATT C ACC
M-CAA	*Mse* I	Primer +3	GAT GAG TCC TGA GTA A CAA
M-CAC	*Mse* I	Primer +3	GAT GAG TCC TGA GTA A CAC
M-CAT	*Mse* I	Primer +3	GAT GAG TCC TGA GTA A CAT
M-CTC	*Mse* I	Primer +3	GAT GAG TCC TGA GTA A CTC
M-CTT	*Mse* I	Primer +3	GAT GAG TCC TGA GTA A CTT
M-CAGT	*Mse* I	Primer +4	GAG TCC TGA GTA A CAGT
M- CACG	*Mse* I	Primer +4	GAG TCC TGA GTA A CACG

3 selective nucleotides (Table 2).

Gel electrophoresis

After completion of PCR, equal volume of 98% formamide dye was added and the samples were then loaded onto 6% polyacrylamide gel. After electrophoresis, the gel was transferred onto Whatman 3, dried and exposed to an x-ray film for 16 h. The film was developed to obtain the autoradiogram.

Data analysis

The amplified fragments (bands) generated using 10-selected primer combinations were scored manually for their presence (denoted as '1') and absence (denoted as '0'). 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 (Table 3) The matrix was used to construct a phenetic dendrogram using the UPGMA (unweighted pair group method of arithmetic averages; Sneath and Sokal, 1973) in order to cluster the samples. The statistical analysis was performed using NTSYS-pc software (version 2.02, Rohlf, 1998).

RESULTS AND DISCUSSION

Due to high degree of reproducibility, AFLP is now increasingly used in the determination of genetic diversity of a large number of wild plant species. In previous studies, AFLP has been used successfully for the reconstruction of the phylogeny of closely related species as well as in the studies of population genetics (Mekanawakul et al., 2003; De Castro et al, 2006; Jian et al., 2006). For the first time, molecular markers (AFLP) have been tested to verify whether they may cast light on the patterns of molecular relationships within Indian *Cycas* species.

The AFLP technique was employed to analyze the 12 sample of six *Cycas* species; *C. annaikalensis* from two populations (IPL03 and IPL 08-new), *C. beddomei* (IPL 08 old and 10) from wild and cultivated plants, *C. circinalis* from two wild populations (IPL02 and 09new), *C. circinalis* var.*orixensis* from four different populations (IPL 04, 06, 13 and 14), C. *swamyi* (IPL09 new) from a single population and *C. zeylanica* (IPL 11) from cultivated plants were used.

Three *Eco*RI +3 primers and five *Mse*l +3 primers were used. After screening, a large number of bands were obtained which was not resoluble. In order to get clearer pattern, the *Mse*l +3 primers (M-CAC, M-CAG) was replaced with *Mse*l +4 primers (M-CACG, M-CAGT), while the *Eco*RI +3 primers were retained. The addition of the extra selective nucleotide reduced the number of amplified bands and the pattern became clearer which made the reading easier. Few bands which were not amplified with the *Mse*l +3 primers were detected with the *Mse*l +4 primers.

The AFLP profiles were generated using two primer combinations, E-AAC –M-CACG and E-AAC x M-CAGT. Primer combination E-AAC –M-CACG detected 46 bands of which 37 were polymorphic (80.4%). Primer combination E-AAC x M-CAGT detected 62 bands of which 41 was polymorphic (66.12%) (Table 4).

A comparison of the genetic similarity between the studied *Cycas* species ranged from 0.46 to 0.87. The least genetic similarity was between *C. circinalis* and *C. zeylanica* (0.46), the greatest similarity was found between *C. circinalis* and *C. annaikalensis* (0.87) (Table 3). The cladistic analysis using AFLP showed that the Indian *Cycas* species used in this study can be broadly divided into two major Clades (A and B). Clade B includes *C. zeylanica*, the insular species, and AFLP cladistic analysis indicates that this species is distinct as compared with Cluster A, which includes all the species growing at the Western Ghats and Eastern Ghats. The result of these clades also corresponds to morphological and anatomical characteristics of vegetative and

Table 3. Mean of similarity matrix of pair wise genetic diversity estimates between Indian *Cycas* species.

Parameter	IPL-02	IPL-03	IPL-04	IPL-06	IPL-08 old	IPL-08 new	IPL-09 old	IPL-09-new	IPL-10	IPL-11	IPL-13	IPL14
IPL-02	1.0000000											
IPL-03	0.8709677	1.0000000										
IPL-04	0.5822785	0.5974026	1.0000000									
IPL-06	0.5802469	0.5949367	0.8493151	1.0000000								
IPL-08 old	0.6923077	0.7142857	0.5810811	0.5789474	1.0000000							
IPL-08 new	0.8281250	0.8548387	0.5696203	0.5679012	0.7031250	1.0000000						
IPL-09 old	0.6075949	0.6025641	0.6962025	0.6309524	0.6081081	0.6363636	1.0000000					
IPL-09-new	0.8750000	0.8153846	0.5679012	0.5662651	0.6716418	0.8030303	0.6538462	1.0000000				
IPL-10	0.6578947	0.6533333	0.5833333	0.6190476	0.6857143	0.6233766	0.5882353	0.6842105	1.0000000			
IPL-11	0.4683544	0.5000000	0.5569620	0.5365854	0.5428571	0.5131579	0.5625000	0.5324675	0.4761905	1.0000000		
IPL-13	0.6351351	0.6301370	0.5238095	0.5595238	0.5694444	0.6666667	0.5662651	0.7083333	0.6973684	0.5454545	1.0000000	
IPL14	0.7571429	0.7536232	0.6049386	0.6625000	0.6666667	0.7681159	0.6097561	0.7857143	0.7236842	0.4938272	0.7746479	1.0000000

reproductive parts and their distinct geographical distribution (Figure 1).

Clade A1 includes *C. circinalis* and *C. annaikalensis* which again confirm their close structural and geographical affinity. *C. beddomei* and *C. circinalis* var. *orixensis* have segregated under the Clade A2, which have clustered together representing their geographical alliance. Both species bear pinnae with characteristic mucilage canals and seed coat with fibrous layer. Geographically, *C. beddomei* is endemic to Cuddapah hills (South Eastern Ghats) and C. *circinalis* var. *orixensis* from Orissa in the North Eastern Ghats. Sub clade A3 includes *C. swamyi* (Figure 1), this species has been considered as a variety of *C. circinalis*, but after a critical evaluation of its habit and morpho-anatomy, it has been identified as a new taxon (Singh and Radha, 2008).

Except *C. beddomei,* all the species of *Cycas* are superficially similar to each other, but close observation on morpho-anatomy of both vegetative and reproductive organs show differentiations. Geographically, also they have not clearly shown any correlation. For example, *C. swamyi*, *C. circinalis* and *C. annaikalensis* are found in the same geographical region, 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. The habitat of *C. swamyi* is quite similar to that of *C. circinalis* of eastern slopes of the Western Ghat. Both grow in open sunny forests; savannas with high temperature might have resulted in some close relationships in their morphology and anatomy. This study shows genetic dissimilarity between *C. circinalis* and *C. swamyi* (0.60 to 0.63) which confirm that *C. swamyi* is different from *C. circinalis.* High degree of variation was observed in *C. circinalis* var. *orixensis* in their wild populations. In this study, materials collected from four different populations were used (IPL 04, 06, 13 and 14). There is a least genetic similarity with the type species, *C. circinalis* (0.58 to 0.67) and within the populations, suggesting that they may be described either as a new species or species complex.

We could not utilize the other species from their wild habitats namely: *C. sphaerica* from Eastern Ghats, *C. pectinata* from north eastern states and *C. rumphii* from Andaman and Nicobar (Insular) in this study. However, on the basis of comparison on morphological, anatomical and molecular analysis, an attempt has been made to identify the phylogenetic groups of *Cycas* of different geographical regions. Since the molecular analysis was based on a limited number of samples; therefore, if, the sampling of larger number of populations of each species is done, perhaps it would have given more convincing result but this exercise requires equally important, monetary input. Even with the limited number of sampling done in AFLP, the results obtained corresponds to the morpho-anatomical studies and on that basis, the authors have divided Indians cycads into four groups viz., Group I- Western Ghats *Cycas*, which includes *C. swamyi*, *C. circinalis* and *C. annaikalensis*; Group II-Eastern

Table 4. Number of bands and polymorphism detected.

S/N	Primer combination	Total band	Monomorphic band	Polymorphic band	Percentage of polymorphic band
1	E-AACx M-CACG	46	9	37	80.4
2	E-AACx M-CAGT	62	21	41	66.12
Total		108	30	78	72.2

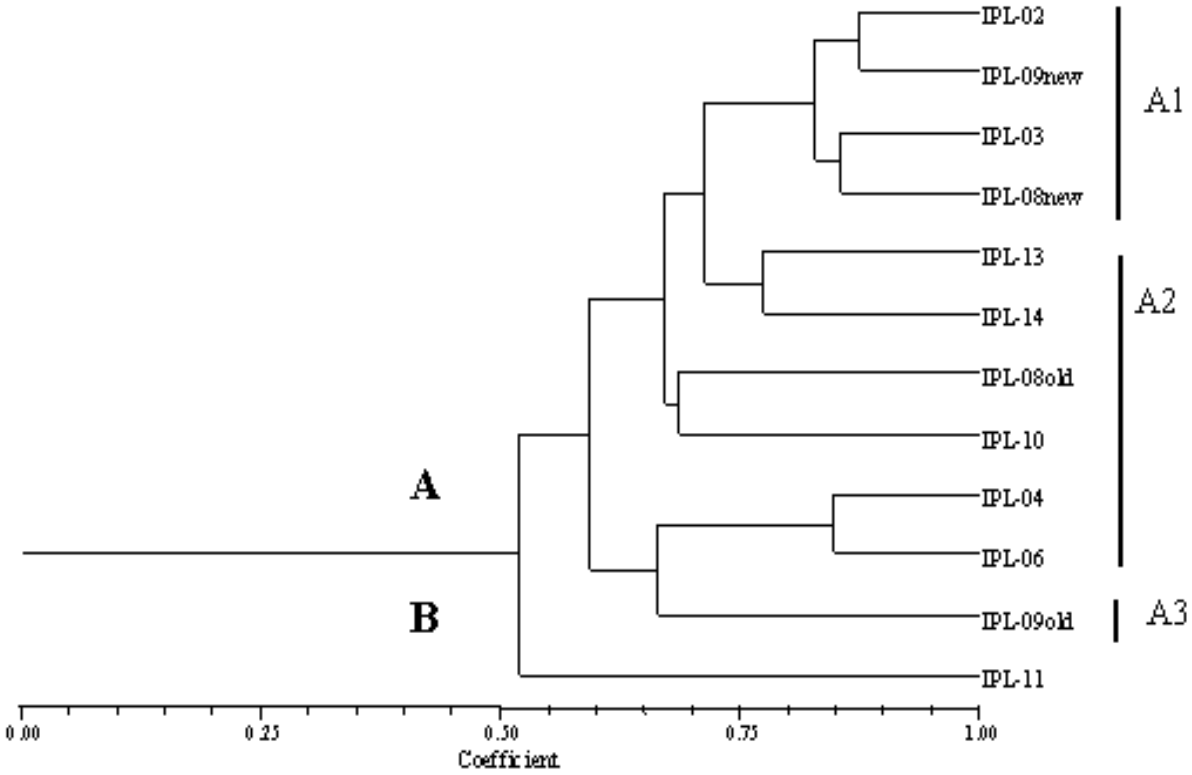

Figure 1. UPGMA cluster analysis of AFLP data for different accessions of Indian *Cycas* species.

Ghats *Cycas* which includes *C. beddomei*, *C. circinalis* var. *orixensis* and *C. sphaerica*; Group III- Indo–Burma, which includes *C. pectinata*; Group IV- Insular *Cycas*, which includes *C. rumphii* and *C. zeylanica*. The present AFLP analysis shows that AFLP markers exhibits a high level of efficiency to detect the DNA polymorphism among the Indian cycads and will be useful to delimitate the species. However, to understand and fully comprehend the biosystematics of all Indian *Cycas* species, it is necessary that maximum possible populations of discovered or yet to be discovered species should be thoroughly investigated.

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