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Molecular Identification of *Cycas* by Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD)

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ABSTRACT

RAPD and RFLP were used to identify nineteen species of *Cycas*. Ten species of these *Cycas* namely *C. chamaoensis*, *C. macrocarpa*, *C. pectinata*, *C. clivicola*, *C. pranburiensis*, *C. litoralis*, *C. tansachana*, *C. siamensis*, *C. nongnoochiae* and *C. simplicipinna* are locally found in Thailand while the nine remaining species of *C. seemannii*, *C. wadei*, C. *bougainvilleana*, *C. chevalieri*, *C. diannanensis*, *C. nathorstii*, *C. edentata*, *C. parvulus* and *C. micholitzii* are from several countries around the world but collectively planted at Nong Nooch Tropical Botanical Garden.

In the RAPD study, twenty random primers were screened to amplify the genomic DNA of nineteen species of *Cycas*. Only five primers, i.e., OPB-1, OPB-8, OPB-14, OPB-15 and OPB-17 of ten nucleotides long were found to give polymorphic DNA patterns. These eighty-seven bands of *Cycas* DNA at the size of 0.35 -2.5 kb could be used to indicate the differences of these *Cycas*. As for RFLP, three probes were synthesized from 5S rRNA gene, 5S rRNA repeat unit gene of *C. clivicola* and 18S rRNA gene of *C. pranburiensis*. The probes were hybridized with the genomic DNA of *Cycas* which had been digested with restriction enzymes *Bam*HI, *Eco*RI and *Dra*I. The phylogenetic trees were constructed based on their similarity index derived from DNA polymorphism of RAPD and RFLP separately. The RAPD data classified nineteen species of *Cycas* into two major groups which mostly corresponded to their geographic origins, i.e., one group of Thailand origin and another of other countries. However, the RFLP data gave a different set of grouping showing more to their morphological characteristics but less on their geographic origins.

Key words: *Cycas*, RFLP, RAPD, phylogenetic tree, geographic origin

INTRODUCTION

Cycads are ancient plants with a long continuity line of heredity. They are classified into 3 families, 11 genera and 250 known species distributed all over the world (Stevenson, 1992) but are mainly found along the intertropical belt, i.e., Africa, India, Indonesia, and North Australia. *Cycas* is the only genus naturally grown in Southeast Asia. However, representatives of different species and genera of Cycad from several distinctive parts of the world are collectively

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grown for academic purpose at Nong Nooch Tropical Botanical Garden, Chonburi province, Thailand.

Although the species of Cycad can be generally identified from corralloid roots, girdlingleaf traces, secondary compounds production or even from the isozymes (Caputo *et al*., 1993), these physico-morphological properties are also affected by the growth conditions and environment which make the closely related species difficult to identify. At present, there are 10 species of morphologically similar *Cycas* found in Thailand but their true genetic identity and evolutionary line are not yet known. To effectively group them and determine their evolutionary relationships, biomolecular technique is needed.

Random Amplified Polymorphic DNA (RAPD) technique is a powerful tool to detect DNA polymorphism and classify closely related species in most plants (Williams *et al*., 1990), and it is considered a fast and easy approach to solve the obscured identification problem. Many kinds of plant have been fingerprinted using RAPD markers (Dettori and Palombi, 2000) to evaluate their phylogenetic relationship, genetic variability and genetic relationships (Lerceteau *et al*., 1997; Ahmad, 1999). On the other hand, Restriction Fragment Length Polymorphism (RFLP) technique makes the use of specific probes from a cDNA or genomic DNA library of the investigated species for the same purpose. This technique has been widely used in studying genetic variation and phylogenetic relationships among populations, species and varieties (Lerceteau *et al*., 1997; Garcia-Mas *et al*., 2000; Sun *et al*., 2001). Since ribosomal DNA (rDNA) are organized in tandem repeat units, they are often used as probes for RFLP. The copy number of these units varies from a few hundred to thousands and are different from species to species (Rogers and Bendich, 1987).

This paper reported on the use of RAPD and RFLP markers to determine the relationship among *Cycas* species and to assess the organization level of genetic diversity through phylogenetic tree.

MATERIALS AND METHODS

Plant materials

Young cycad leaves of nineteen *Cycas* species were collected from the plants grown at Nong Nooch Tropical Botanical Garden, Chonburi, Thailand. These are *C. chamaoensis*, *C. macrocarpa*, *C. pectinata*, *C. clivicola*, *C. pranburiensis*, *C. litoralis*, *C. tansachana*, *C. siamensis*, *C. nongnoochiae*, *C. simplicipinna*, *C. seemannii*, *C. wadei*, C. *bougainvilleana*, *C. chevalieri*, *C. diannanensis*, *C. nathorstii*, *C. edentata*, *C. parvulus* and *C. micholitzii*.

DNA extraction

Genomic DNA was extracted using hexadecetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with some modification. About 0.3 g of young leaves was ground to fine powder in liquid nitrogen. One milliliter of preheated (65°C) 2X CTAB isolation buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 10 mM 2-mercaptoethanol) was added to the sample. The homogenate was incubated at 65°C for 1 h and then extraction was made using one equal volume of chloroform:isoamyl alcohol (24:1). The mixture was centrifuged at 10,000 g for 10 min at room temperature. The aqueous phase was collected and mixed with 1/5 volume of 5X CTAB (5% CTAB and 0.7 M NaCl) and 2/3 volume of isopropanol. The nucleic acid pellet was air-dried and resuspended in 100 mM TE buffer (10 mM Tris, 1mM EDTA, pH 7.0). RNase A was added to the sample at the final concentration of 10 ng/ml. After incubating at 37°C for 30 min, the sample was extracted with phenol:chloroform (1:1). 70% ethanol was added at 2X volume of DNA to make it precipitated. Finally, the DNA pellet was air-dried and resuspensed in 30 ml TE

buffer. DNA pattern and concentration was detected on a 1% agarose gel and by UV spectrophotometer.

RAPD

The protocol for RAPD analysis was adapted from that of Williams *et al*. (1990). The volume of the final reaction (25 μl) consisted of 1 X buffer (10 mM Tris-HCl, pH 8.0 and 50 mM KCl), 3.0 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase, 200 μM dNTP, 10 mM random primer (Operon), 25-100 ng of genomic DNA. Amplifications were made in a Perkin Elmer 9600 thermocycler with an initial denaturing step of 1 min at 94 °C, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final extension of 5 min at 72°C. PCR products were subjected to 1% agarose gel electrophoresis run at 90 V and DNA bands were visualized by ethidium bromide staining.

RFLP

One gram of genomic DNA was cut with the restriction enzymes *Dra*I, *Bam*HI, *Eco*RI, *Hind*III or *stu*I and then subjected to 1% agarose gel electrophoresis. The gels were blotted onto a positively charged nylon membrane (Boehringer Mannheim) by vacuum blotter. The probes were amplified by PCR using 18S rRNA, 5S rRNA and 5S rRNA repeat unit primer and PCR products were labeled with digoxigenin according to the protocol of Dig High prime DNA Labeling and Detection Starter Kit II (Roche). Hybridization was detected by enhanced chemiluminescence on Kodax X-ray film with 0.5-2 h exposure time.

Data analysis

DNA fragments were scored as presence (1) or absence (0) for each primer or restriction enzyme used. These scores were used to calculate their genetic similarity according to Nei and Li (1979), using NTSYS-pc1.80 from which the phenograms were constructed using a UPGMA.

RESULTS AND DISCUSSION

Primer selection and levels of polymorphism

Twenty primers were screened for the genomic DNA amplification of nineteen *Cycas* species. Only 5 primers (Table 1) were found to give polymorphic DNA patterns. The total numbers of 87 bands with the fragments ranging from 0.35- 2.5 kb are shown in Figures 1 and 2. Amplification with primers OPB-1, OPB-8 and OPB-17 revealed unique bands of 0.8 kb, 0.6 kb and 0.7 kb, respectively. These bands could be used as genetic markers to identify the relationships among *Cycas* since they were specific to certain groups of *Cycas* (Nicolosi *et al*., 2000).

RAPD analysis

RAPD data were subjected to UPGMA and NTSYS-pc (Version 1.8). The similarity index showed that the relationships among all nineteen species fell in the range of 0.816-0.516 (Table 2). Maximum similarity was between *C. edentata* and *C. litoralis* (83.9%), while the least similarity were found between *C. wadei* and *C. pranburiensis*, *C. tansachana* and *C. bougainvilleana*, *C. wadei* and *C. bougainvilleana* (50.6 %). The distribution of species within the clusters showed apparent relation with geographical origin. The dendrogram (Figure 3) classified the 19 species into two major clusters, A and B. Cluster A contained all *Cycas* of Thailand geographical origin while cluster B contained those from other countries.

Table 1 Nucleotide sequences of random oligonucleotide primers which showed polymorphism.

Primer no.	Sequence
OPB-1	5′ GTTTCGCTCC 3′
OPB-8	5′ GTCCACACGG 3′
OPB-14	5′ TCCGCTCTGG 3′
OPB-15	5′ GGAGGCTGTT 3′
OPB-17	5′ AGGAACGAAG 3′

Cluster A was further separated into two groups, A I and A II. A I was subdivided into two groups, I and II. Group I consisted of *C. chamaoensis*, *C. macrocarpa*, *C. pectinata*, *C. clivicola* and *C. siamensis*. It is interesting to find that all *Cycas* of group I are of Thailand origin especially *C. chamaoensis*, *C. macrocarpa*, *C. clivicola* and *C. siamensis* were found only in Thailand but *C. pectinata* found also in China, Thailand and Vietnam was somewhat separated as indicated by isozyme (Yang and Meerow, 1996). Group II contained *C. micholitzii*, *C. simplicipinna*, *C. edentata*, *C. litoralis* and *C. pranburiensis*. The similarity index suggested that *C. micholitzii* wass closely related to *C. simplicipinna* which also belonged to the same Stangerioides section based on their morphology (Pu and Chiu, 1990; Stevenson, 1992) (Table 5). The similarity index showed the close relationship of *C. edentata* and *C. litoralis* and both were also

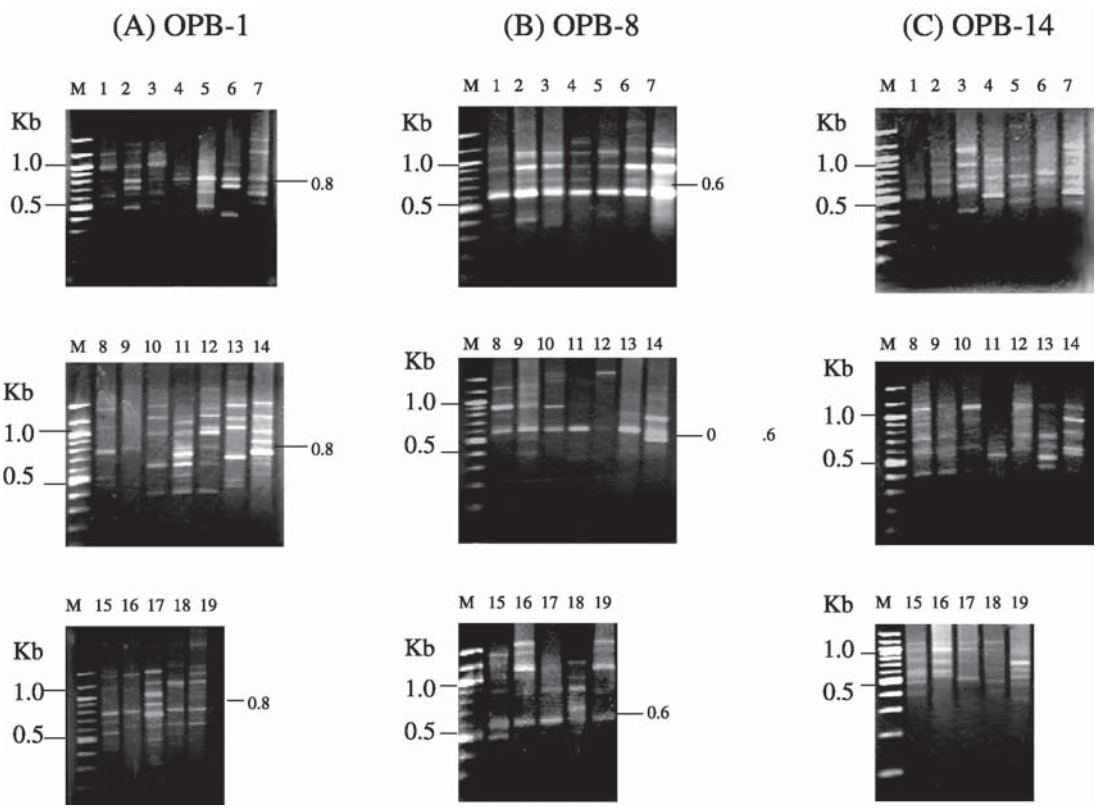

Figure 1 RAPD amplification of 19 *Cycas* species using the primers (A) OPB-1 showing a common band of 0.8 kb, (B) OPB-8 showing a common band of 0.6 kb, (C) OPB-14 having no common band , M: 100 bp DNA size marker, lane 1 *C. chamaoensis*, lane 2 *C. macrocarpa*, lane 3 *C. clivicola*, lane 4 *C. micholitzii*, lane 5 *C. simplicipinna*, lane 6 *C. pectinata*, lane 7 *C. pranburiensis*, lane 8 *C. edentata*, lane 9 *C. litoralis*, lane 10 *C. tansachana*, lane 11 *C. siamensis*, lane 12 C. *nongnoochiae*, lane 13 *C. wadei*, lane 14 *C. seemannii*, lane 15 *C. bougainvilleana*, lane 16 *C. parvulus*, lane 17 *C. chevalieri*, lane 18 *C. nathorstii*, lane 19 *C. diannanensis*. (B) and (C) indicated the similar DNA pattern found between *C. edentata* (lane 8) and *C. litoralis* (lane 9) as well as those of *C. parvulus* (lane 16) and *C. diannanensis*. (lane 19).

in Rumphiae subsection. Group A II comprised of *C. tansachana*, *C. nongnoochiae C. seemannii* and *C. wadei*. The similarity index showed the closer phylogenetic relationships of *C. nongnoochiae* and *C. seemannii* than that of *C. tansachana*. However, morphological identification of *C. nongnoochiae* and *C. tansachana* indicated that these 2 species belonged to the same Indosinenses section and also of Thailand origin (Table 5). It should be noted here that all ten *Cycas* species of Thailand origin were classified into cluster A using RAPD technique and also supported by their morphological characteristics in the close

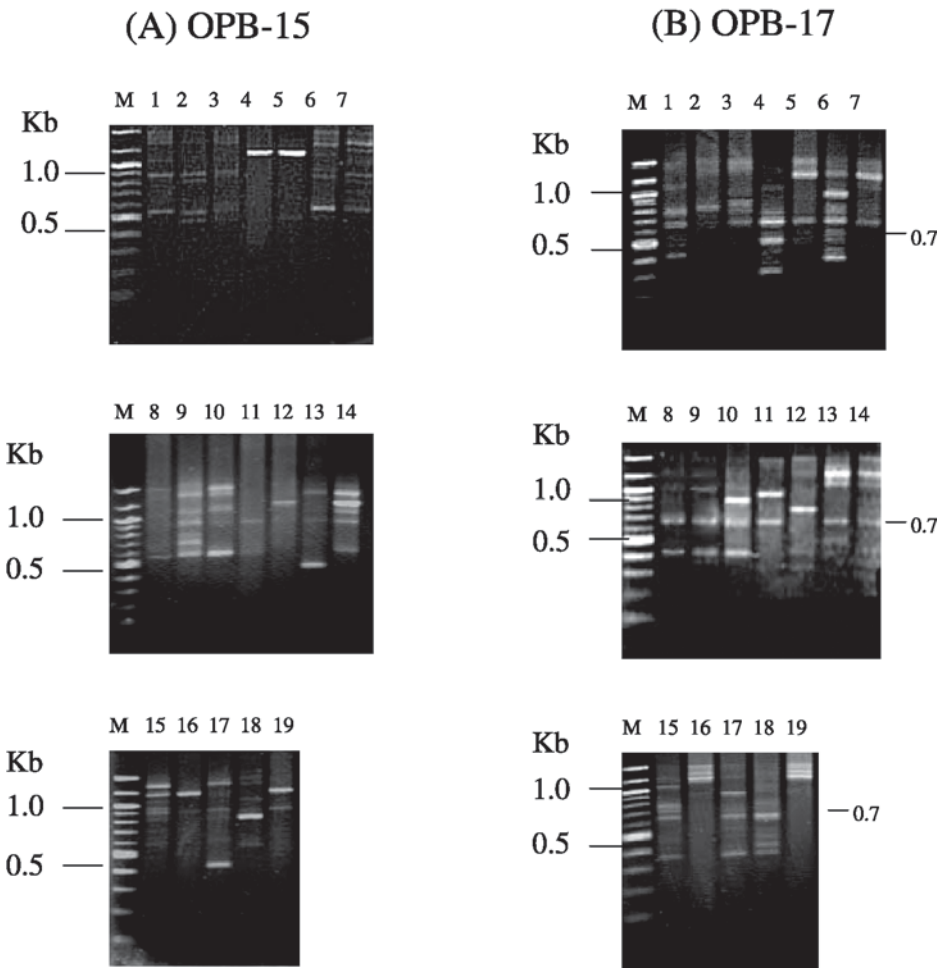

Figure 2 RAPD amplification of 19 *Cycas* species using the primers (A) OPB-15 , (B) OPB-17 showing a common band of 0.7 kb, M: 100 bp DNA size marker, lane 1 *C. chamaoensis*, lane 2 *C. macrocarpa*, lane 3 *C. clivicola*, lane 4 *C. micholitzii*, lane 5 *C. simplicipinna*, lane 6 *C. pectinata*, lane 7 *C. pranburiensis*, lane 8 *C. edentata*, lane 9 *C. litoralis*, lane 10 *C. tansachana*, lane 11 *C. siamensis*, lane 12 C. *nongnoochiae*, lane 13 *C. wadei*, lane 14 *C. seemannii*, lane 15 *C. bougainvilleana*, lane 16 *C. parvulus*, lane 17 *C. chevalieri*, lane 18 *C. nathorstii*, lane 19 *C. diannanensis*. (A) and (B) indicated the similar DNA pattern found between *C. parvulus* (lane 16) and *C. diannanensis* (lane 19).

Table 2 Similarity index of 19 *Cycas* species as identified by RAPD.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.000																		
2	0.816	1.000																	
3	0.747	0.793	1.000																
4	0.713	0.759	0.782	1.000															
5	0.667	0.667	0.667	0.770	1.000														
6	0.770	0.747	0.724	0.690	0.690	1.000													
7	0.724	0.678	0.701	0.713	0.644	0.701	1.000												
8	0.644	0.667	0.690	0.701	0.678	0.621	0.598	1.000											
9	0.713	0.667	0.713	0.770	0.724	0.644	0.667	0.839	1.000										
10	0.644	0.667	0.690	0.678	0.563	0.621	0.552	0.701	0.724	1.000									
11	0.747	0.724	0.793	0.736	0.690	0.678	0.632	0.736	0.736	0.713	1.000								
12	0.667	0.644	0.690	0.724	0.655	0.644	0.575	0.724	0.701	0.678	0.690	1.000							
13	0.621	0.598	0.598	0.586	0.609	0.552	0.506	0.678	0.655	0.678	0.667	0.701	1.000						
14	0.655	0.655	0.632	0.644	0.575	0.563	0.609	0.690	0.690	0.690	0.632	0.713	0.644	1.000					
15	0.609	0.586	0.586	0.667	0.598	0.540	0.609	0.644	0.644	0.506	0.609	0.644	0.506	0.586	1.000				
16	0.621	0.575	0.598	0.770	0.724	0.621	0.575	0.701	0.747	0.632	0.598	0.724	0.609	0.644	0.736	1.000			
17	0.667	0.621	0.575	0.632	0.563	0.575	0.621	0.563	0.632	0.540	0.598	0.655	0.655	0.621	0.736	0.701	1.000		
18	0.701	0.655	0.609	0.690	0.552	0.609	0.609	0.598	0.644	0.575	0.655	0.667	0.598	0.540	0.793	0.644	0.736	1.000	
19	0.621	0.575	0.575	0.701	0.632	0.575	0.552	0.609	0.655	0.655	0.621	0.701	0.586	0.667	0.667	0.816	0.701	0.667	1.000

Note: The names of the nineteen *Cycas* species are shown in Table 5.

relationship of sub-groupings.

Cluster B consisted of *C. bougainvilleana*, *C. nathorstii*, *C. chevalieri*, *C. parvulus* and *C. diannanensis*. The similarity index indicated that *C. bougainvilleana* was closely related to *C. nathorstii* and both are in *Cycas* section (Table 5). The next related one in this group was *C. chevalieri*. As for *C. parvulus* and *C. diannanensis*, they were identified by this method as closely related to one another as also supported by Wang (1996) who grouped them in the same species based on their morphological characteristics of having broad leaflets with flat or undulate margins. All of *Cycas* in cluster B are from other countries.

RFLP analysis

For RFLP, three probes were synthesized from 5S rRNA gene, 5S rRNA repeat unit gene and 18S rRNA gene (Figures 3 and 4). The probes were hybridized with genomic DNA of all nineteen *Cycas* species, which had been digested with restriction enzyme *Bam*HI, *Eco*RI, *Dra*I, *Hind*III and *Stu*I. It was found that three restriction enzymes: *Bam*HI, *Eco*RI and *Dra*I could be used to identify *Cycas* species as also seen in the genetic relationships of *Leymus* species (Anamthawat-Jonsson and Bodvarsdottir, 2001). However, *Hind*III and *Stu*I could not be used to identify the differences among the nineteen *Cycas* species because restriction sites of *Hind*III and *Stu*I might be in the conserved regions on *Cycas* (data not shown).

The results showed 33 polymorphic bands which were further used to construct phylogenetic tree by NTSYS-pc program as

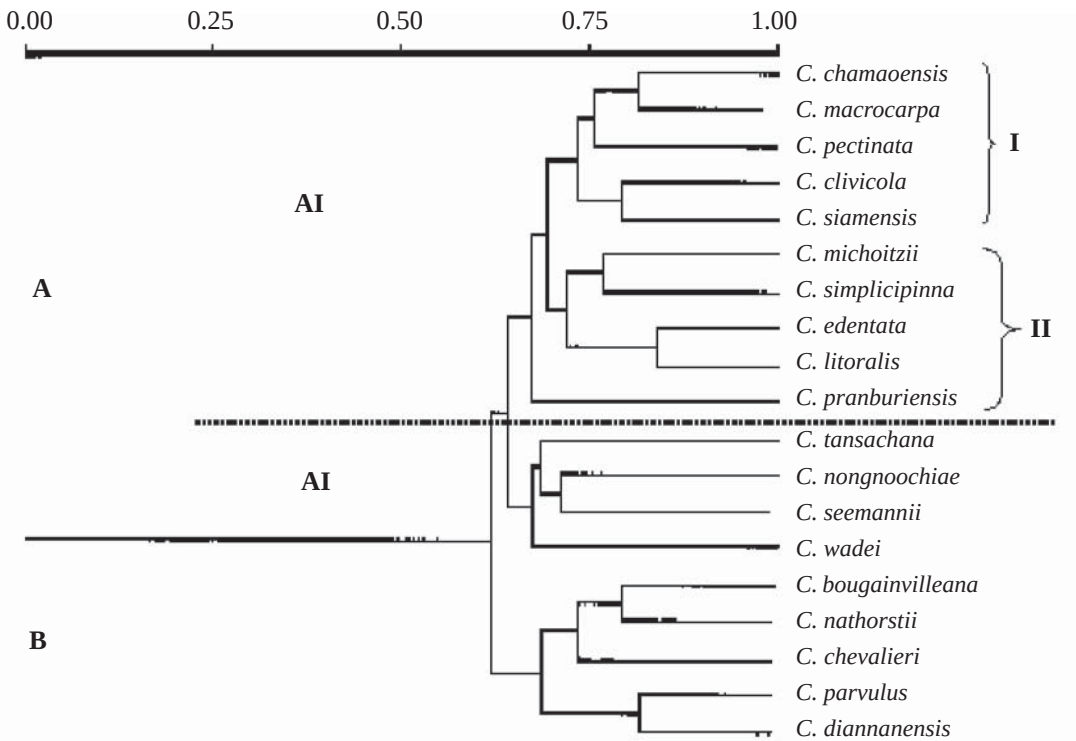

Figure 3 Phylogenetic tree of nineteen *Cycas* species using RAPD technique. The drogram was generated from similarity index based on UPGMA. The species in bold letters were of Thailand origin.

subjected to UPGMA and resulting in the similarity index in the range of 1.0 -0.595 (Table 3). Maximum similarity at 100% was found between *C. wadei* and *C. chevalieri* as well as *C. edentata* and *C. litoralis*. Although both pairs showed 100% similarity, but only *C. edentata* and *C. litoralis* was in accordance with RAPD analysis. The least similarity of 50% were found between *C. bougainvilleana* and *C. pranburiensis*, *C. siamensis* and *C. pranburiensis*, *C. nongnoochiae* and *C. pranburiensis*. The constructed dendrogram also separated nineteen *Cycas* species into two clusters, A and B (Figure 6). Cluster A was further divided into group I, II and III. Group I comprised of *C. chamaoensis*, *C. pectinata* and *C. bougainvilleana*. It was found that *C. pectinata* and *C. chamaoensis* were closely related to each other and both were also in the Indosinenses section based on their morphological classification (Table 5). Group II consisted of *C. macrocapa*, *C. clivicola*, *C. simplicipinna*, *C. edentata*, *C. litoralis* and *C. nathorstii*. The similarity index indicated that *C. macrocapa* and *C. clivicola* were closely related to one another and having the same Thailand origin, while *C. edentata* and *C. litolaris* had very high similarity index which could not be separated from each other and they were also in the same Rumphiae subsection (Table 5). Group III comprised of *C. wadei*, *C. chevalieri*, *C. parvulus*, *C. diannanensis* and *C. seemannii*. However, *C. wadei* and *C. chevalieri* showed very high

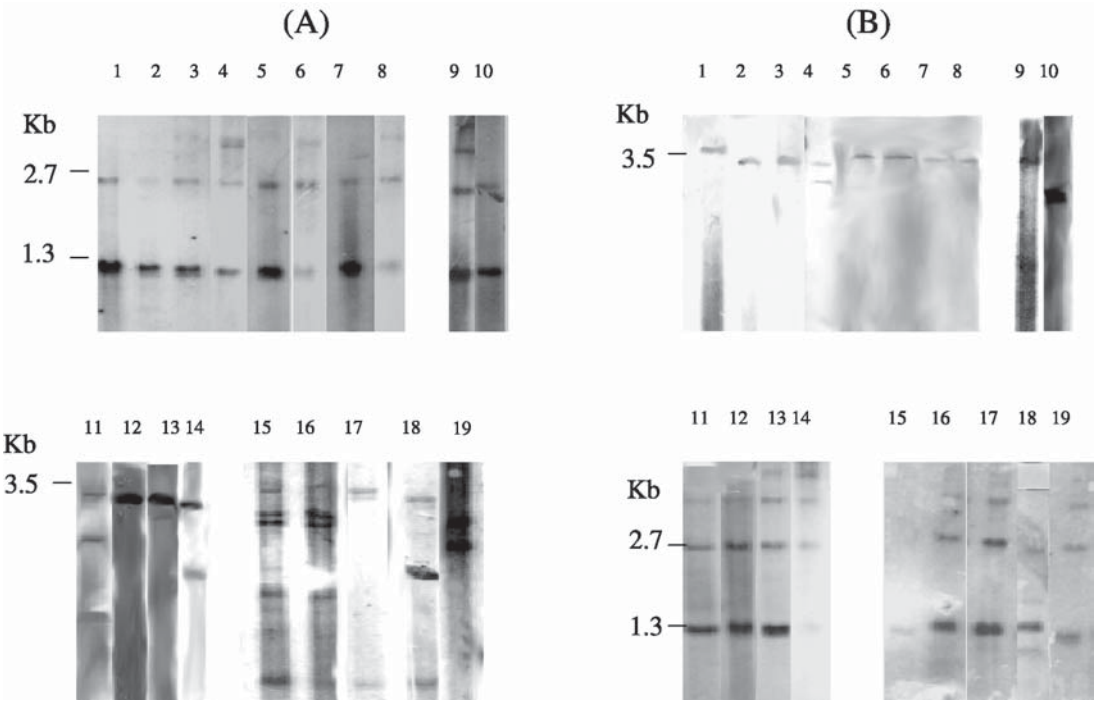

Figure 4 Southern analysis of genomic DNA of 19 *Cycas* species digested with *Bam*HI and hybridized with (A) 18S rRNA and (B) 5S rRNA lane 1 *C. clivicola*, lane 2 *C. macrocarpa*, lane 3 *C. nathorstii*, lane 4 *C. wadei*, lane 5 *C. litoralis*, lane 6 *C. edentata*, lane 7 *C. chamaoensis*, lane 8 *C. seemannii*, lane 9 *C. pranburiensis*, lane 10 *C. simplicipinna*, lane 11 *C. micholitzii*, lane 12 *C. siamensis*, lane 13 *C. nongnoochiae*, lane 14 *C. tansachana*, lane 15 *C. diannanensis*, lane16 *C. parvulus*, lane 17 *C. pectinata*, lane 18 *C. bougainvilleana*, lane 19 *C. chevalieri*.

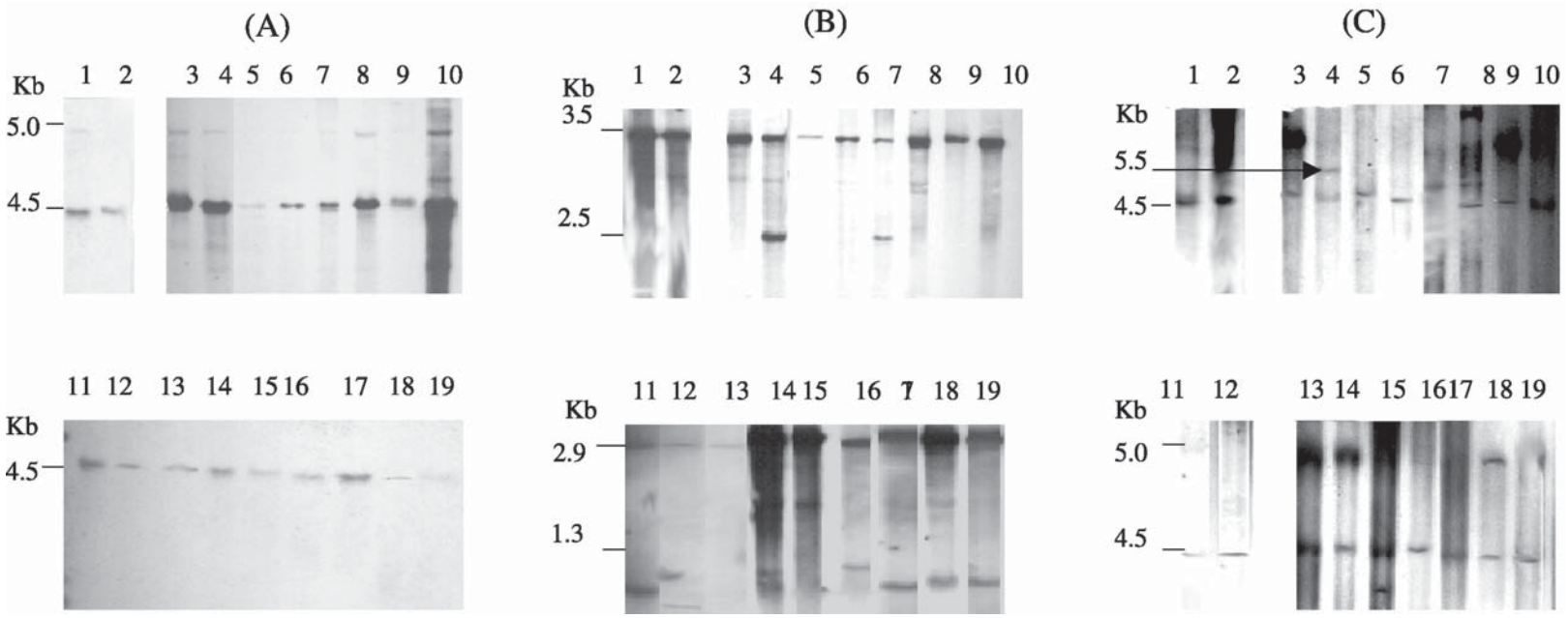

Figure 5 Southern analysis of genomic DNA of 19 *Cycas* species digested with *Dra*I (A) and hybridized with 5S rRNA (B) digested with Dra I and hybridizyed with 18S rRNA lane 1 *C. litoralis*, lane 2 *C. edentata*, lane 3 *C. seemannii*, lane 4 *C. pranburiensis*, lane 5 *C. chevalieri*, lane 6 *C. parvulus*, lane 7 *C. pectinata*, lane 8 *C. diannanensis* , lane 9 *C. bougainvilleana*, lane 10 *C. wadei*, lane 11 *C. siamensis*, lane 12 *C. micholitzii*, lane 13 *C. nongnoochiae*, lane 14 C. *simplicipinna*, lane 15 *C. nathorstii* , lane 16 *C. chamaoensis*, lane 17 *C. tansachana*, lane 18 *C. macrocarpa*, lane 19 *C. clivicola* (C) digested with *Eco*RI and hybridized with 18S rRNA lane 1 *C. chevalieri*, lane 2 *C. parvulus*, lane 3 *C. seemannii*, lane 4 *C. pranburiensis*, lane 5 *C. siamensis*, lane 6 *C. chamaoensis*, lane 7 *C. pectinata*, lane 8 *C. diannanensis*, lane 9 *C. bougainvilleana*, lane 10 *C. wadei*, lane 11 *C. micholitzii*, lane 12 *C. pectinata*, lane 13 *C. litoralis*, lane 14 *C. edentata*, lane 15 *C. simplicipinna*, lane 16 *C. nathorstii*, lane 17 *C. macrocarpa*, *lane* 18 *C. nongnoochiae*, lane 19 *C. tansachana*. *C. pranburiensis* (lane 4) gave a unique 5.5 kb band as shown in (C) and the similar band pattern between *C. litoralis* (lane 13) and *C. edentata* (lane 14).

Tabel 3 The similarity index of 19 *Cycas* species as identified by RFLP data.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.000																		
2	0.765	1.000																	
3	0.824	0.882	1.000																
4	0.706	0.706	0.824	1.000															
5	0.765	0.824	0.765	0.647	1.000														
6	0.882	0.765	0.824	0.647	0.765	1.000													
7	0.647	0.647	0.647	0.529	0.588	0.588	1.000												
8	0.706	0.824	0.706	0.588	0.882	0.765	0.588	1.000											
9	0.706	0.824	0.706	0.588	0.882	0.765	0.588	1.000	1.000										
10	0.824	0.706	0.765	0.706	0.588	0.706	0.529	0.529	0.529	1.000									
11	0.794	0.676	0.735	0.794	0.676	0.676	0.500	0.676	0.676	0.735	1.000								
12	0.735	0.559	0.676	0.676	0.559	0.618	0.500	0.559	0.559	0.853	0.824	1.000							
13	0.794	0.735	0.794	0.676	0.735	0.735	0.735	0.735	0.735	0.618	0.706	0.647	1.000						
14	0.735	0.618	0.618	0.559	0.676	0.735	0.618	0.735	0.735	0.559	0.647	0.588	0.647	1.000					
15	0.853	0.676	0.735	0.618	0.735	0.853	0.500	0.794	0.794	0.676	0.824	0.765	0.706	0.765	1.000				
16	0.735	0.794	0.794	0.676	0.735	0.676	0.735	0.735	0.735	0.618	0.706	0.647	0.824	0.706	0.706	1.000			
17	0.794	0.735	0.794	0.676	0.735	0.735	0.735	0.735	0.735	0.618	0.706	0.647	1.000	0.647	0.706	0.824	1.000		
18	0.765	0.882	0.765	0.647	0.882	0.765	0.647	0.941	0.941	0.588	0.735	0.618	0.794	0.735	0.794	0.794	0.794	1.000	
19	0.706	0.824	0.765	0.647	0.765	0.706	0.647	0.765	0.765	0.588	0.676	0.618	0.794	0.618	0.676	0.912	0.794	0.824	1.000

Note: The names of the nineteen *Cycas* species are shown in Table 5.

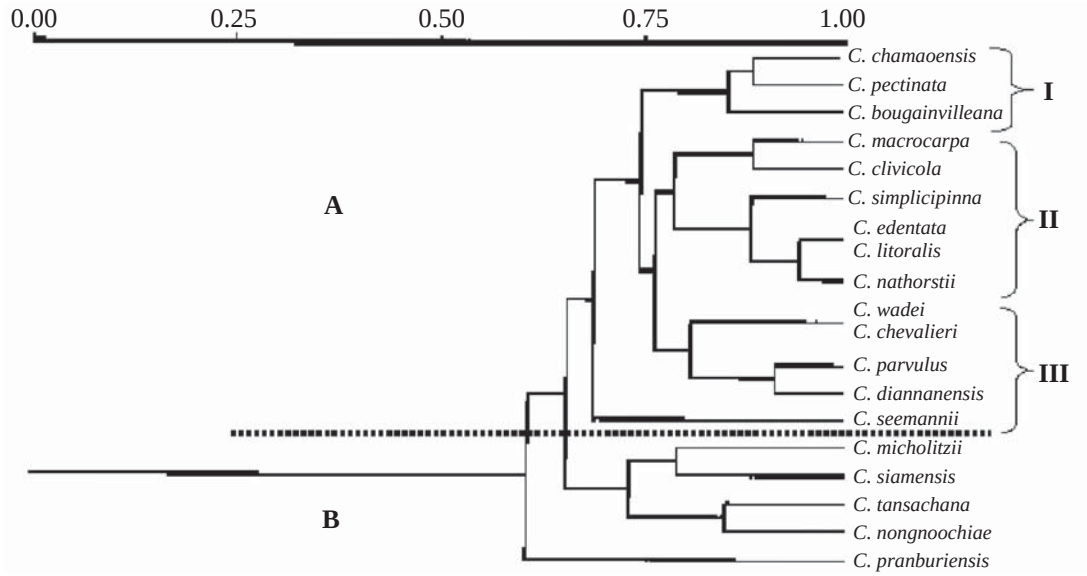

Figure 6 Phylogenetic tree of nineteen *Cycas* species using RFLP technique. The dendrogram was generated from similarity index based on UPGMA. The species in bold letters were of Thailand origin.

similarity index to one another but they were in different sections, i.e., *C. wadei* belonged to Wadeanae section while *C. chevalieri* was in Stangerioides section (Table 5). 5S rRNA gene and 18S rRNA gene of these 2 species might share the same restriction sites, which resulted in the overlapping of the pattern. As for *C. parvulus* and *C. diannanensis* they were closely related as indicated by both their China origin and their belonging to the same Stangerioides section (Table 5).

Group B comprised of *C. micholitzii*, C. *siamensis*, *C. tansachana*, *C. nongnoochiae* and *C. pranburiensis*. It was found that *C. micholitzii* was closely related to *C. siamensis* but they were not in the same morphological section. However, *C. tansachana* and *C. nongnoochiae* were closely related and both were in the same Indosinenses section while *C. pranburiensis* was not found to relate to other eighteen *Cycas* species and was considered an out-group. This was also confirmed by the extra band of 5.5 kb which was unique to only *C. pranburiensis*. The phylogenetic tree derived from RFLP technique, however, illustrated that *Cycas* of Thailand origin were randomly distributed in both cluster A and B.

Levels of polymorphism between RAPD and RFLP

Nineteen *Cycas* species were identified using two different marker systems. The level of polymorphism detected by each marker and their similarity index which indicated their cluster formation are shown in Table 4. For RAPD analysis, only five out of twenty primers were found to give polymorphic pattern among nineteen *Cycas* species. The number of polymorphic bands was 87, giving an average of 17.4 polymorphic bands per total number of primers used. RFLP analyses, on the other hand, three probes were used giving 33 total bands and the average number of polymorphic bands per total number of probes was16.5. Although the average number of polymorphic bands per total number of polymorphic probes/primers obtained from RFLP and RAPD were quite close but the cluster

formation as seen from phylogenetic tree by RAPD data agreed more to the geographic origins while those of RFLP data inclined to support the grouping by morphological characters. The differences found among the dendrogram generated by RAPD and RFLP could be partially explained by the different number of polymorphic bands (87 for RAPD and 33 for RFLP). The high number of RAPD bands may cover more genome than RFLP bands, which cover only the conserved regions. Since the results from RAPD method corresponded well with both their geographic origins and morphology, it may be a more efficient method for identifying different species of *Cycas* than using RFLP.

Table 4 Characteristic of RFLP and RAPD markers used in analyzing the genetic variability of 19 *Cycas* species.

	RAPD	RFLP
Total number of probes/primers	20	3
Number of polymorphic probes/primers	5	2
Total number of bands amplified by polymorphic probes/primers	87	33
Average number of polymorphic bands per total number of polymorphic probes/primers	17.4	16.5

Table 5 The origins and morphological sections of nineteen *Cycas* species.

Number	name	origin	morphological section/subsection*
1	*C. chamaoensis*	Thailand	Indosinenses/Indosinenses
2	*C. macrocarpa*	Thailand	Cycas/Cycas
3	*C. clivicola*	Thailand	Indosinenses/Indosinenses
4	*C. micholitzii*	Vietnam	Stangerioides/Stangerioides
5	*C. simplicipinna*	Thailand	Stangerioides/Stangerioides
6	*C. pectinata*	China, Vietnam and Thailand	Indosinenses/Indosinenses
7	*C. pranburiensis*	Thailand	Cycas/Cycas
8	*C. edentata*	Philippines	Cycas/Rumphiae
9	*C. litoralis*	Thailand, Vietnam, Burma and Malaysia	Cycas/Rumphiae
10	*C. tansachana*	Thailand	Indosinenses/Indosinenses
11	*C. siamensis*	Thailand	Indosinenses/Indosinenses
12	*C. nongnoochiae*	Thailand	Indosinenses/Indosinenses
13	*C. wadei*	Philippines	Wadeanae/-
14	*C. seemannii*	Fiji	Cycas/Rumphiae
15	*C. bougainvilleana*	Papua New Guinea	Cycas/Rumphiae
16	*C. parvulus*	China	Stangerioides/Stangerioides
17	*C. chevalieri*	Vietnam	Stangerioides/Stangerioides
18	*C. nathorstii*	Sri Lanka	Cycas/Cycas
19	*C. diannanensis*	China	Stangerioides/Stangerioides

* classified by Stevenson (1992)

CONCLUSION

The RAPD and RFLP were used to identify nineteen species of *Cycas*. In the RAPD study, only five primers, i.e., OPB-1, OPB-8, OPB-14, OPB-15 and OPB-17 were found to give polymorphic band patterns. RAPD data were subjected to UPGMA and analysis was done by using NTSYS-pc (version 1.8). The similarity index revealed that the species relationships fell in the range of 0.816-0.516. The dendrogram separated nineteen *Cycas* species into two clusters A and B. Cluster A comprised all ten Thailand species of *C. chamaoensis*, C. *macrocapa*, *C. pectinata*, *C. clivicola*, *C. siamensis*, *C. simplicipinna*, *C. litoralis*, *C. pranburiensis*, *C. tansachana*, *C. nongnoochiae* as well as *C. micholitzii*, *C. seemannii*, *C. edentata* and *C. wadei* from other countries. Cluster B comprised of *C. bougainvilleana*, *C. nathorstii*, *C. chevalieri*, *C. parvulus* and *C. diannanensis* all of which are from other countries. The phylogenetic tree of RAPD seems to show good correlation with geographical distribution.

For RFLP, three probes were synthesized from 5S rRNA gene, 5S rRNA repeat unit gene and 18S rRNA gene. The probes were hybridized with genomic DNA of all nineteen species of *Cycas* which had been digested with restriction enzyme *Bam*HI, *Eco*RI and *Dra* I. RFLP data were subjected to UPGMA and analysis was done by using NTSYS-pc (version 1.8). The similarity index showed that the species relationships fell in the range of 1.0-0.595. The constructed dendrogram also separated them into two clusters A and B. Cluster A comprised of *C. chamaoensis*, *C. pectinata*, *C. bougainvilleana*, *C. macrocapa*, *C. clivicola*, *C. simplicipinna*, *C. edentata*, *C. litoralis*, *C. nathorstii*, *C. wadei*, *C. chevalieri*, *C. parvulus*, *C. diannanensis* and *C. seemannii*. Cluster B comprised of *C. micholitzii*, *C. siamensis*, *C. tansachana* and *C. nongnoochiae* while *C. pranburiensis* was not found to relate to eighteen other species and was considered an out-group. The distribution of nineteen *Cycas* species within the dendrogram of RFLP has no apparent relation with the geographical origin but more related to their morphological characteristics.

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