African Journal of Biotechnology Vol.11(4), pp. 743-751, 12 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2835
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

DNA methylation and genetic diversity analysis of genus *Cycas* in Thailand

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Accepted 13 December, 2011

10 *Cycas* species as well as one subspecies localized in Thailand were studied using the methylation sensitive amplification polymorphism (MSAP) technique. 11 MSAP primer combinations were used and 720 MSAP bands were generated. The percentages of DNA methylation estimated from MSAP fingerprints were in the range of 36.05 to 57.44%. MSAP fingerprints were also used for phenogram construction and principal component analysis were based on nucleotide and methylation polymorphisms. Genetic similarity estimated from nucleotide polymorphism among these cycads ranged from 0.86 to 0.92. Cluster analysis using nucleotide and methylation polymorphisms resulted in different groupings for some cycads. The results show that polymorphisms of *Cycas* in Thailand were not only limited in the nucleotide level but also in the pattern of DNA methylation. It was proposed that the percentage and pattern of DNA methylation as well as nucleotide polymorphism were sources of variation in these cycad species.

Key words: *Cycas*, DNA methylation, genetic diversity, methylation sensitive amplification polymorphism (MSAP).

INTRODUCTION

Cycads are ancient seed plants (living fossils) which originated in the Paleozoic era, at least 250 million years ago (Mamay, 1969). They are classified as a gymnosperm. Cycads are woody plants, and form a relatively small group consisting of 185 species in 11 genera, found in the tropical, subtropical and warm temperate regions. Cycads are represented throughout Asia by a single genus, *Cycas*, in the family Cycadaceae. There are approximately 63 *Cycas* species in Asia and only 12 species occur naturally in Thailand. The cycads have been used for ceremonial purposes and they have been planted at burial sites and also have been used as a food source in many regions, including Africa, Asia, Australia and the Americas. Some cycads have been used as an alternative food source in time of deprivation,such as during World War II (Jones, 1998).

Cycads are widely used as ornamental plants and have been regarded as a valuable plant in many countries including Thailand. The cycads have also been recognized as one of the most interesting plants in many kinds of research such as their biology, genetic diversity, evolution and sex determination system. However, the number of plants in the genus *Cycas* has decreased dramatically; two of them are critically endangered (*Cycas chamaoensis* and *Cycas tansachana*), one species is endangered (*Cycas elephantipes*), five species are vulnerable (*Cycas nongnoochiae*, *Cycas pectinata*, *Cycas pranburiensis Cycas macrocarpa* and *Cycas siamensis*) and three species are threatened (*Cycas clivicola* subsp. *clivicola*, *Cycas edentata* and *Cycas simplicipinna*) (Donaldson, 2003). Genetic diversity data at the molecular level of species in the genus *Cycas*, which are useful for species identification and conservation, are very limited. Unlike the flowering plants,

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cycads have never shown much taxonomic or morphological diversity but have the same basic shape. Gorelick and Olsen (2011) speculated that the lack of polyploidy in cycads might be a major cause of their low diversity.

Methylation of cytosine in the 5′ position of the pyrimidinic ring is one of the most important epigenetic modifications of the nuclear DNA in eukaryotes and plays essential roles in regulating gene activity and maintaining genome integrity (Selker, 1997; Rangwala and Richards, 2004). For example, about 30 to 50% of the whole cytosine residues in nuclear DNA are methylated in higher plants, but its level varies in different species (Chan et al., 2005). In plants, cytosine can be methylated at CpG, CpNpG and CpNpN sites, where N represents any nucleotide but guanine. Cytosine methylation is commonly located in the promoter regions or gene first exon (Vallendor et al., 2007) and alters the binding of transcriptional factors and other proteins, preventing RNA–polymerase transcription of DNA and causing gene silencing. The changes in DNA methylation could be influenced by environmental effects resulting in different gene expression and consequently lead to phenotypic differentiation which is beneficial in plant adaptation.

Methylation-sensitive amplification polymorphism (MSAP) is the application of the amplified fragment length polymorphism (AFLP) method based on the different methylation sensitivities of the restriction enzymes, *Hpa*II and *Msp*I (Vos et al., 1995; Jaligot et al., 2002). Both enzymes are isochizomers that recognize the same tetranucleotide CCGG, but have differential sensitivity to methylation at the inner or outer cytosine (Reina-Lopez et al., 1997). The MSAP technique has been used to investigate DNA methylation polymorphisms and to find evidence of epigenetics in many plants such as cotton (Zhao et al., 2008), rice (Sha et al., 2005), palm (Jaligot et al., 2004), banana (Baurens et al., 2003) and watermelon (Nimmakayala et al., 2011).

Plants in the genus *Cycas* are regarded as one of the oldest group of plants which have accumulated changes during their evolutionary processes, for a long time. In addition, living cycads are usually found in the extreme environment that can affect changes in DNA methylation. The information on genetic diversity in these plants is still limited.

The objective of the present study was to investigate DNA methylation polymorphism (MP) as well as nucleotide polymorphism (NP) in some *Cycas* species localized in Thailand using MSAP technique in order to elucidate the role of epigenetics in the genetic diversity of these plants.

MATERIALS AND METHODS

Plant materials and DNA extraction

66 *Cycas* samples consisting of 10 species and one subspecies were collected from the Nong Nooch Tropical Garden, Chonburi province, Thailand. For each species, male and female samples were collected in three replicates for a total of six samples per species (Table 1). Genomic DNA was extracted from the young leaves using the modified method of the International Potato Center (2002).

MSAP procedure

The MSAP protocol was used as described by Kanchanaketu et al. (2007). Restriction and ligation were carried out concurrently and two sets of digestion/ligation reactions of *Eco*RI-*Msp*I and *Eco*RI-*Hpa*II were carried out simultaneously. The pre-selective amplification reaction was performed using *Eco*RI and *Hpa*II/*Msp*I adapter-directed primers (each possessing a single selective base, E+1; HM+1). Polymerase chain reactions (PCRs) were performed with the following profile: 94°C for 60 s, 25 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C and 60 s extension at 72°C and finally 10 min at 72°C to complete extension. Selective amplification (second PCR) of the diluted pre-amplification products was carried out using combination primers E1HM1, E2HM3, E2HM4, E3HM3, H3HM4, E5HM2, E5HM3, E5HM8, E6HM4, E7HM3 and E8HM1 (Table 2). Selective PCR reactions were performed with the following profile: 94°C for 60 s, 36 cycles of 30 s denaturing at 94°C, 30 s annealing and 60 s extension at 72°C, ending with 10 min at 72°C to complete extension. Annealing was initiated at 65°C, which was then reduced by 0.7°C for the next 12 cycles and maintained at 56°C for the subsequent 23 cycles. The second PCR products were separated by 6% denaturing polyacrylamide gel electrophoresis and stained with silver nitrate using the modification method from Benbouza et al. (2006).

Data analyses

MSAP bands were scored as “1” for the presence of bands and “0” for the absence of bands from either *Eco*RI-*Msp*I or *Eco*RI-*Hpa*II digestion. DNA methylation analysis or MP involved comparing the bands obtained from both digestion sets separately. The numbers of each pattern were recorded and used for the estimation of the DNA methylation percentage for each species as described by Xue-Lin et al. (2009). Data from both sets were also interpreted for NP for use in phylogenetic analysis. In both digestion sets, if any band occurred in one species, nucleotide sequences CCGG would be recognized for that species, whereas if no band was presented in any sample of one species, nucleotide sequences CCGG were absent in that species.

A total of 720 markers were analyzed using NTSYSpc Version 2.2k (Exeter software, New York, USA). The band coefficient (Lynch, 1990) and SAHN command were used for the estimation of genetic similarity and cluster analysis, respectively. Cophenetic values and the Maxcomp procedure were used to perform Mantel’s test to determine the goodness of fit for a cluster analysis. The degree of fit can be interpreted as follows:

$0.9 \leq r$: Very good fit
$0.8 \leq r < 0.9$: Good fit
$0.7 \leq r < 0.8$: Poor fit
$r < 0.7$: Very poor fit

The polymorphic information contents (PICs) of each marker were also calculated using

$$PIC = 1 - \Sigma P_i^2$$

Table 1. *Cycas* samples used in the study.

Species	Number	Sex type	Natural habitat
C. chamaoensis (Cha)	1-3, 4-6	M, F	Cliffs and steep slopes on Khao Chamao mountain (eastern Thailand)
C. clivicola subsp. *clivicola* (Cli)	7-9, 10-12	M, F	Limestone cliffs, sea shore, evergreen forest, dry forest (southern Thailand)
C. edentata (Ede)	13-15, 16-18	M, F	Beach forest vegetation above the high tide mark (west and east coasts of Thailand)
C. elephantipes (Ele)	19-21, 22-24	M, F	Seasonally dry deciduous forests, high sand, mainly found nearly the tops of steep slopes or breakaway cliffs stone massif (central Thailand)
C. clivicola subsp. *lutea* (lut)	25-27, 28-30	M, F	Limestone cliffs above moist tropical forest (south-east Thailand)
C. nongnoochiae (Nong)	31-33, 34-36	M, F	Low limestone hills, deciduous forest (central Thailand)
C. pectinata (Pec)	37-39, 40-42	M, F	Hill evergreen forest (western and northern Thailand)
C. pranburiensis (Pran)	43-45, 46-48	M, F	Occurs around Khao Sam Roi Yot Park in dense thickets and full sun on limestone (central Thailand)
C. siamensis (Sia)	49-51, 52-54	M, F	Seasonally dry deciduous forests (central-north Thailand)
C. simplicipinna (Simp)	55-57, 58-60	M, F	Hill evergreen forest (northern Thailand)
C. tansachana (Tan)	61-63, 64-66	M, F	Limestone mountain on cliffs and steep slopes, seasonally dry deciduous forests (central Thailand)

Where, Pi is the frequency of the present or absent allele (Ott, 1991).

Principal component analysis (PCA) was also performed for cluster analysis.

RESULTS AND DISCUSSION

MSAP band patterns and DNA methylation percentages in *Cycas*

From 11 pairs of *Eco*RI+ *Hpa*II/*Msp*I primer combinations used, a total of 720 fragments were amplified from each set. The primer combinations E1HM1, E2HM3, E2HM4, E3HM3, H3HM4, E5HM2, E5HM3, E5HM8, E6HM4, E7HM3 and E8HM1 amplified a total number of 79, 64, 65, 58, 71, 63, 60, 66, 65, 73 and 56 bands, respectively. These fragments could be divided into four types. Type I referred to the loci having no methylation; both *Hpa*II and *Msp*I can cut at CCGG site, resulting in the presence of bands in both sets. Type II referred to loci that were hemi-methylation at the internal cytosine at one strand. The bands were present only when digested with *Hpa*II but not with *Msp*I digestion. Type III, represented the fragments that were presented only in digestion, with *Msp*I but not in *Hpa*II. These loci were fully methylated cytosine at both strands. Type IV referred to the absence of bands in both sets which were excluded from the analysis. The average number of amplified bands from each species was calculated from all six samples and the total methylated bands were calculated from type II and III bands as shown in Table 3.

The average number of bands amplified from each species was calculated. The average number of type I, II and III, and the total bands of all 11 species were 282.39, 69.29, 147.14 and 498.81 bands, respectively. In all species, type I bands were found to be the highest, whereas type II and III bands were found to have lower

Table 2. List of primers and adapters.

Primer/adapter	Sequence
*Eco*RI adapter	5′-CTCGTAGACTGCGTACC-3′ 3′-CATCTGACGCATGGTTAA-5′
*Hpa*II/*Msp*I adapter	5′-GATCATGAGTCCTGAT-3′ 3′-AGTACTCAGGACGAGC-5′
E+A	5′-GACTGCGTACCAATTC**A**-3′
HM+T	5′-ATCATGAGTCCTGCTCGG**T**-3′
E+AAC (E1)	5′-GACTGCGTACCAATTC**AAC**-3′
E+ACG (E2)	5′-GACTGCGTACCAATTC**ACG**-3′
E+ACT (E3)	5′-GACTGCGTACCAATTC**ACT**-3′
E+AAG (E5)	5′-GACTGCGTACCAATTC**AAG**-3′
E+ACC (E6)	5′-GACTGCGTACCAATTC**ACC**-3′
E+AGC (E7)	5′-GACTGCGTACCAATTC**AGC**-3′
E+AGG (E8)	5′-GACTGCGTACCAATTC**AGG**-3′
HM+TAA (HM1)	5′-ATCATGAGTCCTGCTCGG**TAA**-3′
HM+TCC (HM2)	5′-ATCATGAGTCCTGCTCGG**TCC**-3′
HM+TTC (HM3)	5′-ATCATGAGTCCTGCTCGG**TTC**-3′
HM+TTG (HM4)	5′-ATCATGAGTCCTGCTCGG**TTG**-3′
HM+TAG (HM8)	5′-ATCATGAGTCCTGCTCGG**TAG**-3′

Table 3. Average number of bands amplified and average number of total methylated bands of eleven *Cycas* species.

Species	Average number of bands amplified				Average number of total methylated bands	
	Type I	Type II	Type III	Total	Number	Percentage (%)
C. chamaoensis	293.35	62.50	116.20	472.00	178.65	37.86
C. clivicola subsp. *clivicola*	322.30	55.85	138.85	517.00	194.65	37.64
C. edentata	273.50	74.20	135.15	482.85	209.35	43.35
C. elephantipes	330.65	66.65	139.35	536.65	206.00	38.40
C. clivicola subsp. *lutea*	329.20	72.00	120.80	522.00	192.85	36.95
C. nongnoochiae	298.85	63.20	159.50	521.50	222.65	42.70
C. pectinata	285.65	66.85	166.00	518.50	232.85	44.91
C. pranburiensis	200.80	68.65	201.00	470.50	269.65	57.45
C. siamensis	218.00	82.00	163.00	463.00	245.00	52.90
C. simplicipinna	245.00	80.00	144.00	469.00	224.00	47.77
C. tansachana	309.00	70.35	134.70	514.00	205.00	39.88
Average	282.39	69.29	147.14	498.81	216.42	43.61

numbers. The average number of total methylated bands was estimated from the type II and type III bands and ranged from 178.65 to 269.65 with an average of 216.42 band. The percentage of DNA methylation in each species ranged from 36.95 to 57.45% and could be classified into three groups. First, the low methylated species (those having a percentage of methylation lower than 40%) consisted of *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes*, *C. clivicola* subsp. *lutea* and *C. tansachana*. Second, the moderate methylated group (those having a percentage of methylation from 40 up to (and including) 50%) consisted of *C. edentata*, *C. nongnoochiae*, *C. pectinata* and *C. simplicipinna*. The last group was the highly methylated species (having a percentage of methylation more than 50%) and they consisted of *C. siamensis* and *C. pranburiensis*. *C. pranburiensis* had the highest percentage of DNA methylation (57.44%) and could be distinguished from the

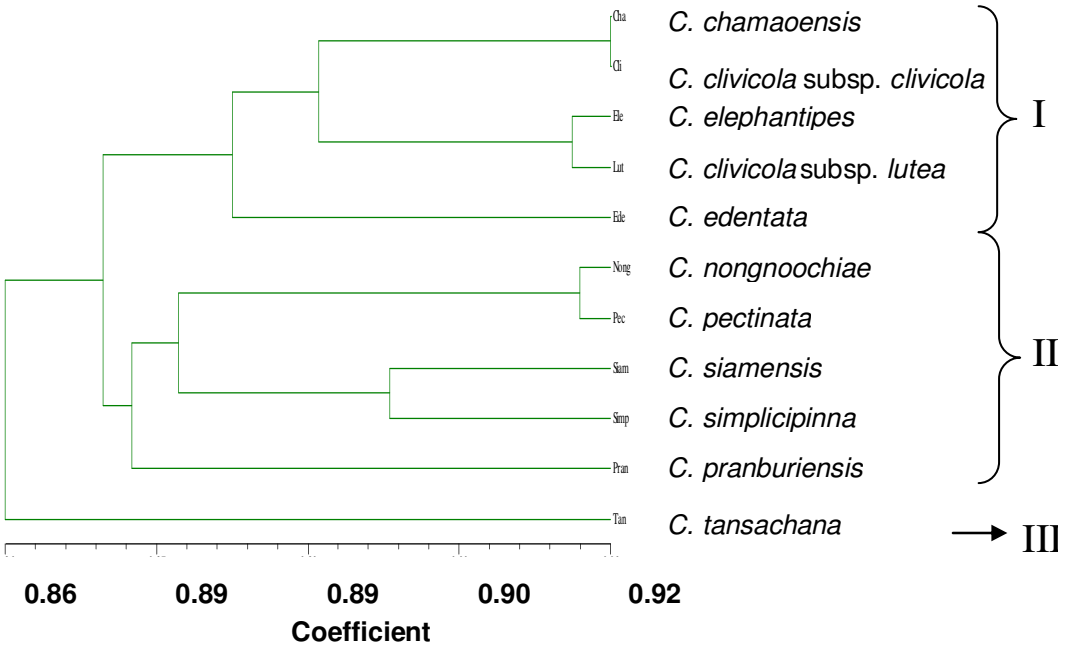

Figure 1. Phenogram of 11 *Cycas* species constructed using nucleotide polymorphism (NP) data.

other *Cycas* species by its short leaves with a small number of broad, coriaceous leaflets and mostly unarmed petioles, and the elongate, gently curving and tapering sterile microsporophyll apex, as described by Yang et al. (1999).

Genetic diversity analysis and methylation diversity analysis

From 11 MSAP primers, 720 markers were analyzed as having NP and used to estimate the genetic diversity among the *Cycas* species. The polymorphic information contents of the markers ranged from 0 to 0.5 with an average of 0.15. Genetic similarity among the samples estimated by the method of Lynch (1990) was found to range from 0.86 to 0.92. Cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) could classify 11 *Cycas* species into three main groups with a genetic similarity of 0.867 (Figure 1).

The first group consisted of *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes*, *C. clivicola* subsp. *lutea* and *C. edentata*. Interestingly, these four *Cycas* species have a percentage of methylation lower than 40% except for *C. edentata* that has 44.48% of methylated DNA. It has been reported that *C. clivicola* subsp. *lutea* was closely related to *C. clivicola* subsp. *clivicola* but was found in different locations (Tang et al., 1997). From the current study, the genetic similarity between these two cycads was 0.89 whereas the percentage of DNA methylation was almost the same. *C. chamaoensis* and *C. clivicola* subsp. *lutea* were difficult to identify because these cycads were reported to have the same morphological characters and also to be found in the same natural environment (Tang et al., 1997). However, the genetic similarity between these cycads from the current study was 0.88. The most distinct species of the first group, *C. edentata*, also had a higher percentage of DNA methylation when compared with the rest of the species in this group. *C. edentata* or *Cycas litoralis* were found near the seashore or on cliffs near the river which was different from the other species.

The second group comprised of *C. nongnoochiae*, *C. pectinata*, *C. siamensis*, *C. simplicipinna, C. pranburiensis, C. pectinata* and *C. siamensis*. It has been reported that *C. pectinata* and *C. siamensis* were clustered in the same group called the *C. pectinata* complex (Norstog and Nicholls, 1997). From the current study, genetic similarity between these two cycads was 0.88. The DNA methylation percentage of *Cycas* species in this group ranged from 44.90 to 57.45. The most distinct cycad of this group was the highest methylated, *C. pranburiensis*. The third group contained only *C. tansachana* samples. The cophenetic correlation coefficient (Mantel test) revealed a very good fit of cluster analysis, with a value of r = 0.964 for the dendrogram. Cluster analysis using UPGMA was consistent with PCA. Principal components 1, 2 and 3 accounted for 58.62, 7.03 and 6.03% of the total variation, respectively (Figure 2).

To estimate the epigenetic diversity based on the methylation site, data of MP from *Eco*RI-*Msp*I and *Eco*RI-

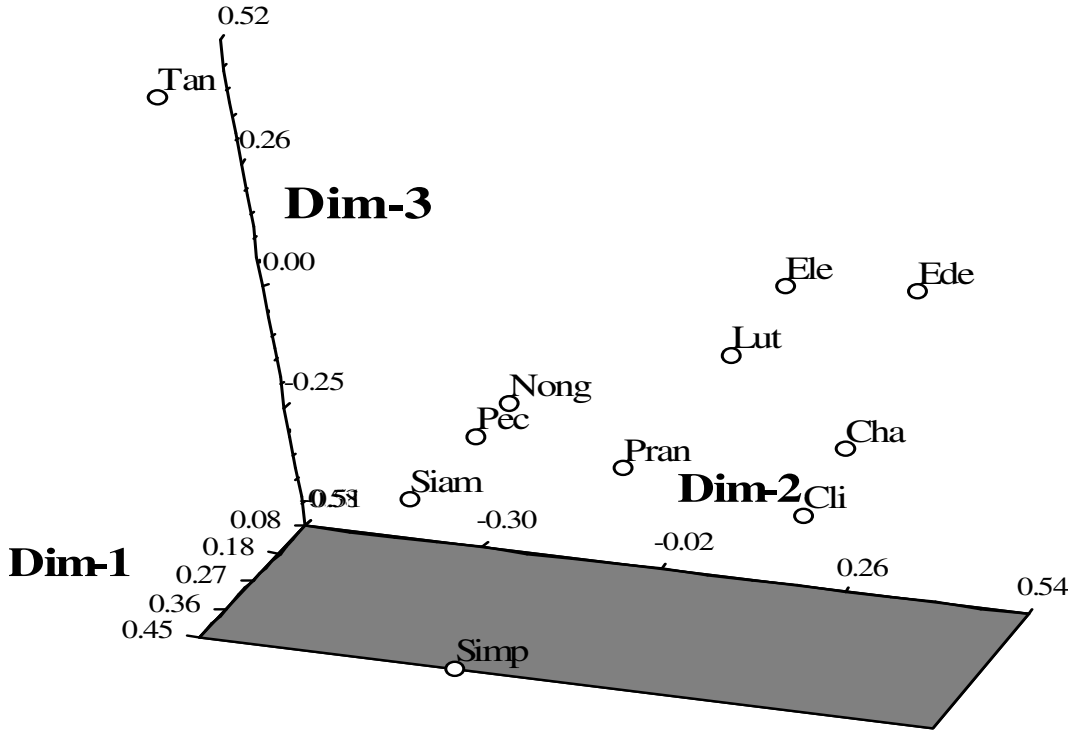

Figure 2. Principal component analysis of 11 *Cycas* species analyzed from nucleotide polymorphism (NP) data.

*Hpa*II digestion were used to construct the phylogenetic tree and PCA. UPGMA phenograms were also constructed to compare the patterns of genetic relationship based on nucleotide polymorphism with epigenetic diversity. The estimation based on *Msp*I polymorphisms revealed that the genetic similarity of the cycads ranged from 0.78 to 0.94, whereas the estimation based on *Hpa*II polymorphisms showed a range of 0.79 to 0.95. The principal components one, two and three from *Hpa*II polymorphisms accounted for 11.59, 7.84 and 6.47% of the total variation, respectively. The principal components one, two and three from *Msp*I polymorphisms accounted for 10.96, 8.33 and 5.46% of the total variation, respectively. The PCA analysis using methylation polymorphism showed lower values for PCA 1, 2 and 3 and indicated higher levels of methylation variation in these *Cycas* species when compared with polymorphisms at the nucleotide level.

Cluster analysis of the *Cycas* species based on *Msp*I polymorphism could be classified into three main groups (Figure 3a). The first group comprised *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes*, *C. clivicola* subsp. *lutea* and *C. edentata*. The second group consisted of *C. nongnoochiae*, *C. pectinata* and *C. tansachana*. The last group consisted of *C. simplicipinna*, *C. siamensis* and *C. pranburiensis*. Cluster analysis based on *Hpa*II polymorphisms could be classified into four main groups (Figure 3b). The first group comprised *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes*, *C. clivicola* subsp. *lutea* and *C. edentata*. The second group comprised only *C. tansachana*. The third group consisted of *C. nongnoochiae*, *C. pectinata* and *C. pranburiensis*. The last group consisted of *C. simplicipinna* and *C. siamensis*.

The results from cluster analysis of both methylation sensitive polymorphisms are in agreement with cluster analysis from NP data for the group of *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes*, *C. clivicola* subsp. *lutea* and *C. edentata*. However, some changes in clusters were obtained from MP analysis. First, *C. tansachana* was clustered with *C. pectinata* and *C. nongnoochiae* in *Msp*I polymorphism analysis whereas in *Hpa*II polymorphism analysis, *C. tansachana* was separated as a specific group. Second, *C. pranburiensis* was clustered with *C. siamensis* and *C. simplicipinna* in *Msp*I polymorphism analysis whereas in *Hpa*II polymorphism analysis, *C. tansachana* was clustered with *C. pectinata* and *C. nongnoochiae*. These changes in cluster analysis indicated the variation of DNA methylation pattern among the studied cycads. Moreover, cluster analysis from MP data also revealed the possible effects of environmental habitat in *C. edentata*. *C. edentata* was clustered with *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes* and *C. clivicola* subsp. *lutea*

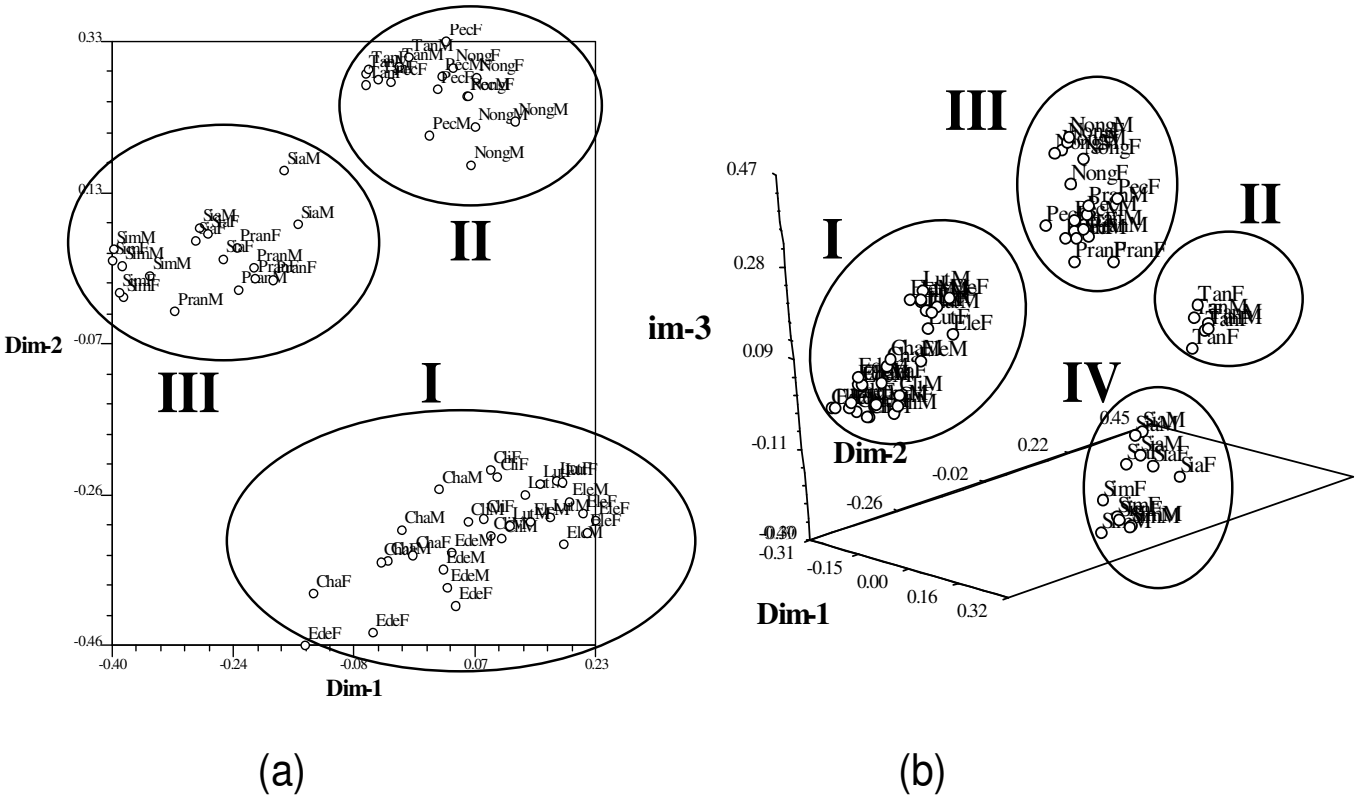

Figure 3. PCA cluster analysis of 66 *Cycas* samples based on MP data of *Eco*RI + *Msp*I (a) and *Eco*RI + *Hpa*II (b). MP, Methylation polymorphism.

in all types of analysis. However, in *Msp*I clustering, the group of *C. edentata* samples was slightly separated from the major group. Among the major group, *C. edentata* was the only cycad that was usually found near the seashore, while the rest of the cycads in this group were located in mountainous areas. From the current study, the MSAP fingerprint showed that *C. edentata* did not only have nucleotide polymorphism but also methylation polymorphism when compared with the group of *C. chamaoensis*, *C. clivicola* subsp. *clivicola*, *C. elephantipes* and *C. clivicola* subsp. *lutea.*

The cluster analysis from the two types of methylation polymorphism showed different clustering results and indicated that there was variation in the patterns of DNA methylation among the cycads studied. These results suggest that during the evolutionary process, each *Cycas* species accumulated some epialles for a period of time and these epialles were faithfully transmitted, which could have resulted in different percentages and variations in the pattern of DNA methylation in each species. The differences in the methylation level and the pattern of methylation are important in determining the levels of gene expression which can lead to the differences in gene expression that confer phenotypic effects (Kalisz and Purugganan, 2004; Anger et al., 2010).

DNA methylation has been known to be one of the important plant adaptation processes. Generally, a plant genome has 30 to 50% methylation at the cytosine base (Xue-Lin et al., 2009). DNA methylation plays an important role for many processes in plants such as gene regulation, plant development and gene imprinting (Finnegan et al., 1998). The differences in DNA methylation percentage were possibly caused by adaptation to specific environment habitats (Finnegan, 2001). DNA methylation could turn genes on or off in the appropriate environments to promote plant survival. The DNA methylation pattern is also transmitted through meiosis during DNA replication by the maintenance DNA methylation enzyme, DNMT1. However, the non-methylated sequences could be methylated by a *de novo* methylation process which could be induced by intrinsic signals or environmental signals (Finnegan et al., 1998). *Cycas* species in Thailand are usually found in limestone mountainous areas, seasonally dry deciduous forests and in a drought environment. Some of them are located near rivers or other water sources. Cycads living in different types of habitat also show different morphological characters such as leaf and stem shape and growth habit. Wang et al. (2011) revealed that drought stress could induce genome-wide DNA methylation changes

which account for 12.1% of the total site-specific methylation differences in the rice genome.

Among the cycad species in this study, *C. simplicipinna* (the only *Cycas* species that is usually found in cool mountain forest) has a unique leaf morphology with leaves being bright-to-deep green and highly glossy and longer compared with other species (Hill and Yang, 1999). However, from the current study, the percentage of DNA methylation of this species is not significantly different from other species and *C. simplicipinna* is separated with *C. siamensis* in all cluster analysis. This is very interesting because *C. simplicipinna* and *C. siamensis* are clearly different in their distributions, habitats and morphological characters. However, these two species are distributed within the same geographical boundary and they also have the specific subterranean stem that is absent from other species found in Thailand (Tang et al., 1997). The genetic similarity from NP analysis of the current study between these two species is 0.895 whereas the average genetic similarities estimated from *Eco*RI-*Msp*I and *Eco*RI-*Hpa*II data are 0.80 and 0.82, respectively.

In conclusion, the present study focused on the level of DNA methylation and both the nucleotide and methylation polymorphisms of cycads in Thailand. The percentage of DNA methylation in the *Cycas* species of Thailand was estimated and the effects of DNA methylation were also speculated using cluster analysis of the methylation-based phenogram and PCA. The average percentages of DNA methylation were obtained from male and female plants of each species, indicating that the percentage was different among the studied cycads with a range of 36.05 to 57.44%. Cluster analysis of 11 cycads from NP and MP data resulted in the same grouping in the majority of studied species. A genetic similarity based on NP and MP data was found to range from 0.82 to 0.92 and 0.78 to 0.95, respectively. The results obtained indicate low to moderate diversity of *Cycas* species in Thailand. The principal components estimated from NP and MP data showed that variation in DNA methylation was much higher than nucleotide variation. The present study shows that *Cycas* species in Thailand not only have nucleotide polymorphisms but also methylation polymorphisms as well as an amount of DNA methylation, which could be involved in the evolutionary process of the genus *Cycas*.

ACKNOWLEDGEMENTS

The authors would like to thank the Nongnooch Tropical Garden, Chonburi province, for providing all the *Cycas* specimens used in this research. The suggestions and review of the manuscript by Associate Professor Dr. Amara Tongpan were appreciated. This work was supported by the Kasetsart University Research and Development Institute (KURDI) and a grant from the Graduate School, Kasetsart University, Thailand.

REFERENCES

Anger B, Castonguay E, Massicotte R (2010). Environmentally induced phenotypes and DNA methylation: How to deal with unpredictable conditions until the next generation and after. Mol. Ecol. 19: 1283-1295.

Baurens FC, Bonnot F, Bienvenu D, Causse S, Legavre T (2003). Using SD-AFLP and MSAP to assess CCGG methylation in the banana genome. Plant Mol. Biol. Rep. 21: 339-348.

Benbouza H, Jacquemin JM, Baudoin JP, Mergeai G (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnol. Agron. Soc. Environ. 10(2): 77-81.

Chan SWL, Henderson IR, Jacobsen SE (2005). Gardening the genome DNA methylation in *Arabidopsis thaliana*. Nat. Rev. Genet. 6: 351-360.

Donaldson J (2003). Cycads. Status survey and conservation action plan. IUCN, Gland, Switzerland and Cambrige, UK.

Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998). DNA methylation in plants. Annu. Rev. Plant. Physiol. Plant. Mol. Biol. 49: 223-247.

Finnegan EJ (2001). Epialleles- a source of random variation in times of stress. Curr. Opin. Plant. Biol. 6: 101-106.

Gorelick R, Olson K (2011). Is lack of cycad (Cycadales) diversity a result of lack of polyploidy? Bot. J. Linn. Soc. 165: 156-167.

Hill KD, Yang SL (1999). The genus *Cycas* (Cycadaceae) in Thailand. Brittonia, 51(1): 48-73.

Jaligot E, Beule T, Rival A (2002). Methylation-sensitive RFLPs: Characterization of two oil palm markers showing somaclonal variation-associated polymorphism. Theor. Appl. Genet. 104: 1263-1269.

Jaligot E, Beule T, Baurens FC, Billotte N, Rival A (2004). Search for methylation-sensitive amplification polymorphisms associated with the “mantled” variant phenotype in oil palm (*Elaeis guineensis* Jacq.). Genome, 47: 224-228.

Jones DL (1998). Cycads of the World. Reed. New Holland, Sydney.

Kanchanaketu T, Hongtrakul V, Sangduen N (2007). Analysis of sex determination in some Cycads using methylation-sensitive amplification polymorphism (MSAP). Kasetsart J. 41: 641-650.

Kalisz S, Purugganan MD (2004). Epialleles via DNA methylation: Consequences for plant evolution. Trends Ecol. Evol. 9(6): 309-314.

Lynch M (1990). The similarity index in DNA fingerprinting. Mol. Biol. Evol. 7: 478-484.

Mamay SH (1969). Cycads: fossil evidence of late Paleozoic origin. Science, 164: 295-296.

Nimmakayala P, Vajja G, Gist RA, Tomason YR, Levi A, Reddy UK (2011). Effect of DNA methylation on molecular diversity of watermelon heirlooms and stability of methylation specific polymorphisms across the genealogies. Euphytica, 177: 79-89.

Norstog KJ, Nicholls TJ (1997). The Biology of the Cycads. Cornell University Press. New York. p. 363.

Ott J (1991). Analysis of Human Genetic Linkage. John Hopkins University Press. Baltimore, Maryland.

Rangwala SH, Richards ET (2004). The value-added genome: building and maintaining genomic cytosine methylation landscapes. Curr. Opin. Genet. Dev. 14: 686-691.

Reina-Lopez GR, Simpson J, Ruiz- Herrera J (1997). Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphism. Mol. Genet. Genomic, 253: 703-710.

Selker EU (1997). Ephigenetic phenomena in filamentous fungi: Useful paradigms or repeat induced confusion? Trends Genet. 13: 296-301.

Sha AH, Lin XH, Huang JB, Zhang DP (2005). Analysis of DNA methylation related to rice adult plant resistance to bacterial blight based on methylation-sensitive AFLP (MSAP) analysis. Mol. Gen.

Genomic, 273: 484-490.
Tang W, Yang S, Vatcharakorn P (1997). Cycads of Thailand. Nong Nooch Tropical Garden. p. 33.
The International Potato Center (CIP) (2002). Molecular Biology Laboratory Protocols.[Available Source: http://www.cipotato.org/training/Materials/Molecular/Molecular.htm]. [Cited: April 2004].
Valledor L, Hasbu'n R, Meijo'n M, Rodri'guez JL (2007). Involvement of DNA methylation in tree development and micropropagation. Plant Cell. Tissue Organ Cult. 91: 75-86.
Vos P, Hogers R, Bleeker M, Reijans A, Pot J, Peleman J, Kuiper M, Zabean M (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414.
Wang WS, Pan Y, Zhao X, Dwivedi D, Zhu L, Ali J, Fu B, Li Z (2011). Drought-induced site-specific DNA methylation and its association with drought tolerance in rice (*Oryza sativa* L.). J. Exp. Bot. 62(6): 1951-1960.
Xue-Lin L, Zhong-Xu L, Yi-Chun N, Xiao-Ping G, Xian-Long Z (2009). Methylation-sensitive amplification polymorphism of epigenetic changes in cotton under salt stress. Acta Agron. Sin. 35(4): 588-596.
Yang SL, Tang W, Hill KD, Vatcharakorn P (1999). *Cycas pranburiensis* (Cycadaceae), a new cycad from Thailand. Brittonia, 51(1): 44-47.
Zao Y, Yu S, Xing C, Fan S, Song M (2008). Analysis of DNA methylation in cotton hybrids and their parents. J. Biol. Mol. 42(2): 169-178.