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Analysis of Sex Determination in Some Cycads Using Methylation-Sensitive Amplification Polymorphism (MSAP)

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ABSTRACT

This is the first report on the analysis of sex determination in cycads using MSAP technique. Prior works using cytogenetic techniques, molecular markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) did not clearly identify sex in cycads. This modified AFLP technique using isochizomer enzyme (*Msp*I and *Hpa*II) called methylation-sensitive amplification polymorphism (MSAP) was carried out to assess the pattern of cytosine methylation in both sexes of *Cycas* and *Zamia*. Using seven pairs of primers, 364 bands some of which showing sex-specific were produced and classified into three groups. The first group was non-polymorphic markers, whereas the second group was chosen from the results of differentation ability of *Msp*I and *Hpa*II to cut the methylated sequences, but sex-different markers were not obtained. Markers in the third group were methylation-sensitive and they also showed some polymorphic patterns between the two sexes. We suggested that sex in cycads may be associated with DNA methylation, but further study is required to reach a conclusive result.

Key words: *Cycas*, *Zamia*, sex determination, DNA methylation**,** MSAP

INTRODUCTION

Cycads are small groups of plants with many unique features; they are generally regarded as living fossils of the plant kingdom (Treutlein and Wink, 2002). Cycads have been instrumental in our understanding the evolution of angiosperms and gymnosperms because they have recognizable morphological characteristics intermediate between less-recently existed plants such as ferns and more-evolved plants such as angiosperms (Brenner *et al*., 2003). The living cycads can be divided into three families; Cycadaceae, Stangeriaceae and Zamiaceae which consisted of 11 genera, 297 species and sub-species. All cycads are dioecious. They appear to possess little if any sexual dimorphism as vegetative structures are concerned. Therefore, one can hardly distinguish the sexes of a species by visual inspection unless their reproductive organs are present. Little is known regarding sex determination in cycads although there have been several reports on sex in cycads, but none is conclusive (Norstog and Nicholls, 1997).

Sexually reproducing plants and animals can be classified into two groups by the fundamental distinction of whether all individuals are essentially alike in their gender condition which is called sexually monomorphic (e.g., monoecious, hermaphrodite) and those in which

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there are different kinds of individuals such as separated male and female which is called sexually polymorphic (e.g., dioecious). In several plants, sex determination is controlled by sex chromosomal system (Charlesworth, 2002). Some well-studied plants such as white campion (*Silene latifolia*), hop (*Humulus lupulus*) and Sorrel species (*Rumex sp*.) are referred to as model species in this regard (Negrutiu *et al*., 2001, Vyskot and Hobza, 2004). Molecular markers such as random amplified polymorphic DNA (RAPD) were also used to identify sex in nutmeg (Shibu *et al*., 2000), *Actinidia deliciosa* var. *deliciosa* (Shirkot *et al*., 2002), *Eucommia ulmoides* Oliv. (Xu *et al*., 2004) and *Carica papaya* L. (Urasaki *et al*., 2002).

Although there are many investigations in sexually reproducing organisms, there is still no single unified hypothesis explaining sex determination (Gorelick, 2003), but it is clear that sexual dimorphism is a late developmental decision during the life cycle of the plant (Negrutiu *et al*., 2001). Differences in nucleotide sequences might not be a cause of sex determination, but other mechanism could be responsible for this.

Epigenetic mechanisms, especially DNA methylation, are known to have an important role in the control of gene expression (Finnegen *et al*., 1998). Data showing the different DNA methylation levels at every stages of plant development was reported (Vyskot *et al*., 1995). Several works indicated that DNA methylation play some parts in various processes of plant development (Drozdenyuk *et al*., 1976; Munksgaard, 1995; Finnegen *et al*., 2000), but little work was on the role of DNA methylation in sex determination mechanism. DNA methylation also plays a role in genomic imprinting — the different expression of gene inherited from maternal and paternal gametes, which does not occur only in mammalian embryo and its derivatives, but also in flowering plants (Finnegen *et al*., 2000). Janousek *et al*. (1996) studied the epigenetic control of sexual phenotype in *Melandrium album* by treating seeds with hypomethylating drugs and the result showed that the one treated with 5-azacytidine induced a sex change to androhermaphroditism which then produced fertile male and female gametes in about 21% of male plants, while no apparent phenotypic effect was observed in female plants. From this result it might imply that the level of DNA methylation could have some effects on sex determination but there are not enough data to support this speculation.

Many techniques were developed to detect the changes in the level of DNA methylation; one is the application of amplified fragment length polymorphism (AFLP) called methylation-sensitive amplification polymorphism (MSAP). This technique is based on the use of the two isochizomers, *Hpa*II and *Msp*I, that are different in their sensitivity to methylation of the recognition sequences. Both enzymes recognize the tetra-nucleotide sequence 5′-CCGG-3′, but the action is determined by the methylation status of the external or internal cytosine residues. *Hpa*II is inactive when either one or both of the two cytosines is fully-methylated (both strands methylated) but cleaves to the hemi-methylated sequence (only one strand methylated), while *Msp*I cleaves hemi- or fully-methylated $C^{5m}CGG$, but not $^{5m}CCGG$. MSAP was first developed to determine DNA methylation events in dimorphic fungi (Reyna-Lopez *et al*., 1997) and later adapted for the detection of cytosine methylation in the rice genome (Xiong *et al*., 1999), pepper (Portis *et al*., 2004), apple (Xu *et al*., 2000) and Siberian ginseng (Chakrabarty *et al*., 2003).

Here, we reported the evaluation of pattern and extent of cytosine methylation between male and female cycads using methylation-sensitive amplification polymorphism (MSAP) together with the pooled DNA analysis technique and also demonstrated that sex determination mechanism in cycads may be associated with DNA

methylation mechanism.

MATERIALS AND METHODS

Plant materials and DNA extraction

The total of 24 samples collected from twelve diploid cycads comprising six male and female of both *Cycas* species (*C. chamaoensis*, *C. clivicola*, *C. edentata*, *C. elephantipes*, *C. pectinata* and *C. siamensis*) and also six male and female of *Zamia* species (*Z. amblyphyllidia*, *Z. herrarea*, *Z. inermis*, *Z. integrifolia*, *Z. loddigesii* and *Z. pumila*) were used in this study. The young leaves from Nong Nooch Tropical Garden, Chonburi province, Thailand, were collected. DNA was extracted from the young leaves using the modified method from Molecular Biology Laboratory Protocols (http://www.cipotato.org/training/Materials/Molecular/Molecular.asp).

MSAP procedure

The modified Bulk Segregant Analysis technique was used to reduce the polymorphic bands from each species and to facilitate clear identification of the sex-associated markers. DNA was pooled into 4 groups; male *Cycas*, female *Cycas*, male *Zamia* and female *Zamia*. The MSAP protocol was adapted from Reyna-Lopez *et al*. (1997) and Portis *et al*. (2004). Restriction and ligation were done concurrently and two sets of digestion/ligation reactions were carried out simultaneously. In the first reaction, 100 ng of genomic DNA was digested with 10 U of *Eco*RI (Fermentas) plus 10 U of *Msp*I (Fermentas) in the final volume of 50 µl containing digestion reaction buffer (33mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA), 5 pmol of *Eco*RI adapter, 25 pmol of *Hpa*II/*Msp*I adapter, 5 U of T4 DNA ligase and ligation reaction buffer (33mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA). The mixture was then incubated at 37°C for 5 h. The reaction was stopped by incubating at 65°C for 10 min and diluting 10 times in 0.1xTE (1mM Tris-HCl, 0.1mM EDTA, pH8) for PCR amplification. The second reaction was carried out as above, except *Hpa*II (Fermentas) was used instead of *Msp*I.

The pre-selective amplification reaction was performed using 2 µl of diluted digestion/ligation reactions, in 25 µl of PCR reaction mixture containing 200 mM Tris-HCl pH 8.4, 500 mM KCl, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pmol of *Eco*RI and *Hpa*II/*Msp*I adapter-directed primers (each possessing a single selective base, E+1; HM+1) and 1 U of *Taq* DNA polymerase (Invitrogen). PCR reactions were performed with the following profile: 94°C for 60 sec, 25 cycles of 30 sec denaturing at 94°C, 30 sec annealing at 55°C and 60 sec extension at 72°C, and finally for 10 min at 72°C to complete extension. After checking the presence of a smear of fragments (100–1000 bp in length) by agarose electrophoresis, the amplification product was diluted 40 times in 0.1xTE.

Selective amplification (second PCR) of the diluted pre-amplification products was carried out using a total of 12 primer combinations obtaining from four *Eco*RI primers and three *Hpa*II/*Msp*I primers plus three selective bases of each primer(E+3, HM+3) listed in Table 1. Selective PCR reactions were performed with the following profile: 94°C for 60 sec, 36 cycles of 30 sec denaturing at 94°C, 30 sec annealing and 60 sec extension at 72°C, ending with 10 min at 72°C to complete extension. Annealing was initiated at the temperature of 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 mixed with 10 µl of loading dye (98% formamide, 10mM EDTA, 0.01% w/v bromophenol blue and 0.01% w/v xylene cyanol), denatured at 95°C for 5 min and separated by electrophoresis on 6% denaturing polyacrylamide sequencing gels (6% polyacrylamide 29:1, 7M Urea) in 1× TBE buffer.

Table 1 List of primers and adapters used.

Primers/adapters	Sequences
*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	5′-GACTGCGTACCAATTC**AAC**-3′
E+ACG	5′-GACTGCGTACCAATTC**ACG**-3′
E+ACT	5′-GACTGCGTACCAATTC**ACT**-3′
E+AGT	5′-GACTGCGTACCAATTC**AGT**-3′
HM+TAA	5′-ATCATGAGTCCTGCTCGG**TAA**-3′
HM+TCC	5′-ATCATGAGTCCTGCTCGG**TCC**-3′
HM+TTC	5′-ATCATGAGTCCTGCTCGG**TTC**-3′

The gels were pre-run at 300 V for about 30 min before 10 μl of the mixture was loaded. Gels were run at 400 V for about 2 h, and stained with silver nitrate using the modification method from Caetano-Anolles (1997). The results were analyzed by comparing 4 lanes of each genus (male digested with *Eco*RI+*Msp*I(EM), female digested with *Eco*RI+*Msp*I(EM), male digested with *Eco*RI+*Hpa*II(EH) and female digested with *Eco*RI+*Hpa*II(EH), respectively). All bands were scored as "1" for the presence of bands and "0" for the absence of bands as exemplified in Figure 1.

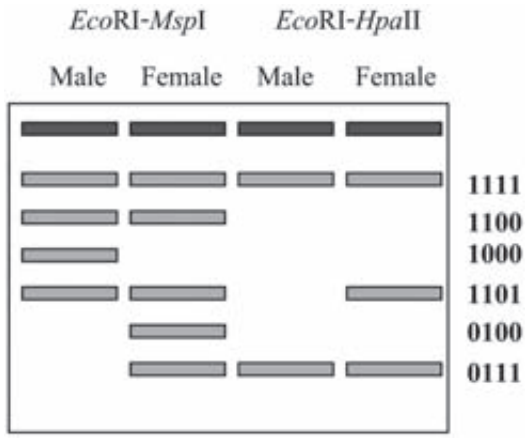

Figure 1 Example of some patterns obtained from 7 primer pairs scored as "1" for the presence of bands and "0" for the absence of bands.

RESULTS AND DISCUSSION

Having twelve primer pairs screened, seven primer pairs produced clear bands were selected for further MSAP analysis. A total of 364 markers generated from the seven pairs of primer were classified into three groups. In the first group, the bands were presented in all four lanes (1111). These bands were most frequently observed and represented approximately 70% of the total number of MSAP bands. Although there was no polymorphism between the two sexes in this group, some generic specific bands were observed. Nineteen markers were specific to genus *Cycas* and eleven markers were specific to *Zamia*. The second group (13 markers) showed polymorphic bands between those digested with *Eco*RI+*Msp*I and *Eco*RI+*Hpa*II**.** This pattern was the result from the differentiation ability of enzymes *Msp*I and *Hpa*II to digest different methylated sequences, but it did not show polymorphism between sexes (1100, 0011). The third group was found to show

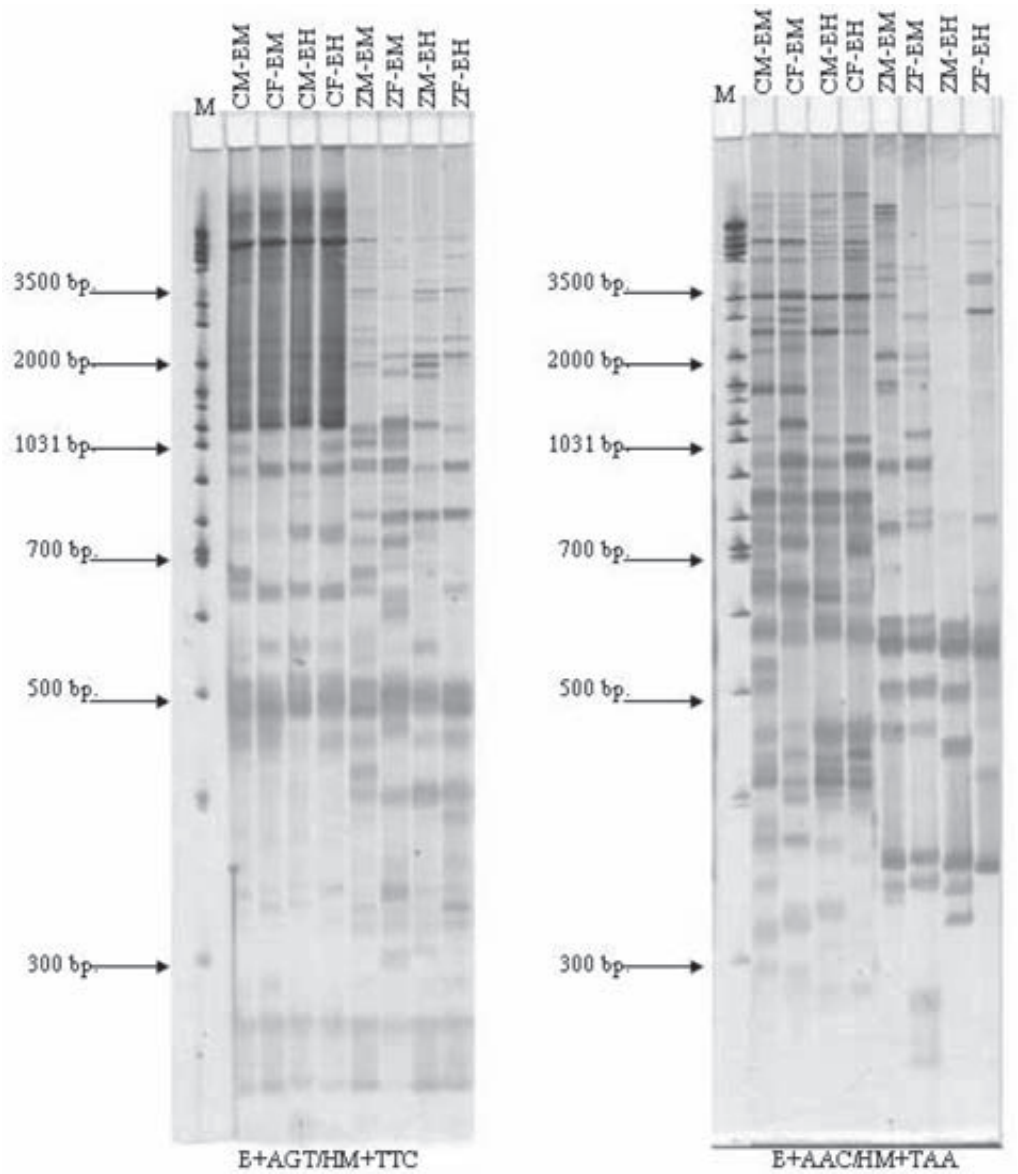

Figure 2 MSAP profile from E+AGT/HM+TTC and E+AAC/HM+TAA primers.
CM: pooled DNA of male *Cycas*; CF: pooled DNA of female *Cycas*
ZM: pooled DNA of male *Zamia*; ZF: pooled DNA of female *Zamia*
EM: digestion with enzyme *Eco*RI and *Msp*I; EH: digestion with *Eco*RI and *Hpa*II
M: DNA ladder mix (Fermentas)

polymorphism and appeared to be the result of different DNA methylation events occurred in male and female cycads. Ninety-nine markers in this group revealed 12 patterns as shown in Table 2. Genus *Cycas* had 39 markers, whereas genus *Zamia* had 60 markers.

MSAP analysis using *Msp*I and *Hpa*II was determined at the CCGG recognition site. There were several patterns of DNA methylation in the CCGG sequence. By comparing the AFLP

Table 2 Patterns of MSAP markers in the third group showing polymorphism as indicated by the different DNA methylation events between male and female cycads.

Haplotype No.	Pattern	Number of patterns found		
		Cycas	*Zamia*	Total
1	0001	6	6	12
2	0010	2	7	9
3	0100	6	10	16
4	0101	1	2	3
5	0110	2	1	3
6	0111	0	2	2
7	1000	11	13	24
8	1001	1	3	4
9	1010	3	5	8
10	1011	0	5	5
11	1101	5	3	8
12	1110	2	3	5
	Total	39	60	99

patterns obtained after digestions with *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II, the following four classes of DNA fragments were assigned (1) fully-methylated at the internal cytosine: only *Msp*I could cleave the $C^{m}CGG$ sequence (designated as M-type) (2) hemi-methylated at the external cytosine: only *Hpa*II could digest $^{hm}CCGG$ sequence (designated as H-type) (3) fully-methylated at both cytosines: $^{m}C^{m}CGG$ and fully-methylated at the external cytosine: $^{m}CCGG$. Neither *Msp*I nor *Hpa*II enzyme could cleave $^{m}C^{m}CGG$ and $^{m}CCGG$ (designated as X-type) and (4) non-methylated: CCGG. Both *Msp*I and *Hpa*II could digest CCGG sequence (designated as HM-type).

From twelve polymorphic patterns shown in Table 2, we could evaluate possible DNA methylation in male and female cycads from four classes as mentioned above and they could be grouped into four cases (Table 3). In the first case (61 markers), one sex was fully-methylated at both cytosines (X-type), while another sex was either fully-methylated at the internal cytosine (M-type) or hemi-methylated at the external cytosine (H-type). In the second case (11 markers), one sex was fully-methylated at both cytosines (X-type) whereas another sex gave only non-methylated sequence (CCGG, HM-type). The third case (20 markers), one sex gave non-methylated sequence (CCGG, HM-type) whereas another sex was fully-methylated at the internal cytosine (M-type) or hemi-methylated at the external cytosine (H-type). The fourth case (7 markers), one sex was fully-methylated at the internal cytosine (M-type) whereas another sex was hemi-methylated at the external cytosine (H-type). It was found that female cycads had more methylated pattern of $^{m}C^{m}CGG$ or $^{m}CCGG$ and $^{hm}CCGG$ than those of male cycads whereas $C^{m}CGG$ pattern occurred in male more than in female cycads. The number of markers also indicated that there was less non-methylated sequence (CCGG) in male than those of female.

There are many techniques used for detecting DNA methylation level in organisms (Wojdacz and Hansen, 2006). Here, methylation-sensitive amplification polymorphism (MSAP) was used to assess methylation events in male and female cycads. MSAP, as described by Reyna-Lopez *et al*. (1997) and Xiong *et al*. (1999) is a

Table 3 Evaluation of possible DNA methylation status in each sex from MSAP markers.

Pattern	Possible DNA methylation events		Case
	Male	Female	
0001	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	$^{hm}CCGG$ (H)	1
0010	$^{hm}CCGG$ (H)	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	1
0100	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	$C^{m}CGG$ (M)	1
1000	$C^{m}CGG$ (M)	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	1
0101	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	CCGG (HM)	2
1010	CCGG (HM)	$^{m}C^{m}CGG$ or $^{m}CCGG$ (X)	2
1011	CCGG (HM)	$^{hm}CCGG$ (H)	3
1101	$C^{m}CGG$ (M)	CCGG (HM)	3
1110	CCGG (HM)	$C^{m}CGG$ (M)	3
0111	$^{hm}CCGG$ (H)	CCGG (HM)	3
0110	$^{hm}CCGG$ (H)	$C^{m}CGG$ (M)	4
1001	$C^{m}CGG$ (M)	$^{hm}CCGG$ (H)	4

Note: $C^{m}CGG$ is fully-methylated at internal cytosine (M-type)
$^{hm}CCGG$ is hemi-methylated at external cytosine (H-type)
CCGG is a non-methylated sequence (HM-type)
$^{m}C^{m}CGG$ is fully-methylated at both cytosines (X-type)
$^{m}CCGG$ is fully-methylated at external cytosine (X-type)

Table 4 The amount of MSAP markers in male and female cycads for each methylation status.

Methylation status	*Cycas*		*Zamia*	
	Male	Female	Male	Female
$^{m}C^{m}CGG$ or $^{m}CCGG$	13	16	18	25
$^{hm}CCGG$	4	7	10	14
$C^{m}CGG$	17	10	19	14
CCGG	5	6	13	7
Total	39	39	60	60

combination between using the methylation-sensitive enzymes and AFLP technique. The advantages of MSAP is having no requirement for prior genome information, while giving high number of existing methylation events using only a relatively small number of primer combinations and the additional ability to clone and characterize novel methylated sequences (Peraza-Echeverria *et al*., 2001). The commonly used enzymes in MSAP are *Msp*I and *Hpa*II, having CCGG as the recognition site. *Hpa*II is inactive if either one of cytosine is fully-methylated but it will cut the hemi-methylated sequence in which only one DNA strand is methylated, whereas *Msp*I can cleave $C^{m}CGG$ but not $^{m}CCGG$.

Digestion of genomic DNA with *Eco*RI/*Msp*I or *Eco*RI/*Hpa*II results in three classes of DNA fragments: 1) *Eco*RI-*Eco*RI, 2) *Eco*RI-*Msp*I or *Eco*RI-*Hpa*II and 3) *Msp*I - *Msp*I or *Hpa*II – *Hpa*II. However, in the amplification conditions applied, only *Eco*RI–*Hpa*II or *Eco*RI–*Msp*I fragments could be preferentially amplified since the *Hpa*II/*Msp*I primer has a lower annealing temperature than the *Eco*RI primer (Portis *et al.*,2004). The 'less frequent cutter' *Eco*RI recognizes the hexa-nucleotide site 5′-GAATTC-

3′ and has an average of one recognition site per 4kb; while the 'frequent cutter' *Msp*I/*Hpa*II recognizes the tetra-nucleotide site of 5′-CCGG-3′ and has one recognition site per 256 bp. Therefore, each *Eco*RI recognition site is flanked with an average of 16 *Msp*I/*Hpa*II recognition site. If the next neighboring *Msp*I/*Hpa*II recognition site is non-methylated, the resulting *Eco*RI-*Msp*I and *Eco*RI-*Hpa*II fragments are identical, and thus common AFLP bands will appear in all four lanes. On the other hand, if the next neighboring *Msp*I/*Hpa*II recognition site is methylated, polymorphic *Eco*RI-*Msp*I and *Eco*RI-*Hpa*II DNA fragments are generated (Xu *et al*., 2000).

Analysis of sex in 6 *Cycas* and 6 *Zamia* species resulted in three groups of DNA bands. The first and second groups, which represented more than 70% of all markers, were not sex polymorphic bands. Ninety-nine markers in the third group, which were clustered into 12 patterns, appeared to show the different methylation status in male and female cycads. However, these 12 patterns could also be categorized into two groups. The first group was 0001, 0010, 0100, 0101, 1000 and 1010 patterns. These patterns showed polymorphism between sexes that might be due to two reasons, i.e., different methylation status and also differences in nucleotide sequences because bands were present only in one sex. For example in pattern 0001, bands occurred only in female E-H lane but not in female E-M lane. This indicated that in this region, female methylation status was ^{hm}CCGG**.** However, neither male E-M nor male E-H showed any bands at this same region which, therefore, indicated that male had methylation status of either ^{m}C^{m}CGG or ^{m}CCGG, or there were some point mutation occurred in the recognition site**.** So this polymorphism could not be only considered as methylation differences but also nucleotide differences. Janousek *et al*. (1996) concluded that female sex suppression in *Melandrium album* XY male was dependent on methylation of specific DNA sequences. While DNA methylation level decreased in male, the male was changed into androhermaphrodite in which female gametes were fertile. The possible methylation pattern might be either 0010 or 0001 in which males were fully-methylated (both enzymes could not cut) but females were less-methylated (one of the two enzymes could cut). Our results of the methylation patterns in the first group, therefore, supported the results of Janousek *et al*. (1996) but the role of nucleotide sequence should also be highly considered as well. In the second group (0110, 0111, 1001, 1011, 1101 and 1110), however, the bands were present at least in one lane for both male and female. So polymorphism in this group was methylation-sensitive and certainly not from nucleotide sequences differences. For example in pattern 0110, male was supposed to be ^{hm}CCGG because the bands presented only in E-H lane and the female should be C^{m}CGG because the bands occurred only in E-M lane, which could be implied that in this region male and female had different DNA methylation status. This was also true for the remaining five methylation patterns (0111, 1001, 1011, 1101 and 1110) in the second group as well.

However, there were two major constraints associated with this MSAP technique. First, this method could only investigate the presence of cytosines present within the recognition sites of the isoschizomers which represented only a small proportion of the cytosines in the genome, e.g., 5′-CCGG-3′ in the present study, while the expected frequency of this sequence in the genome was in the order of 1/256. Second, the technique could not detect some cytosine methylation in the restriction sites. As previously mentioned, *Hpa*II was inactive at fully-methylated cytosines but it could cut the hemi-methylated sequence in which only one DNA strand was methylated, whereas *Msp*I could cleave C^{5m}CGG but not ^{5m}CCGG. Although differences between banding patterns obtained following

digestion with *Hpa*II and *Msp*I are the result of different cytosine methylation in the restriction sites, these two enzymes could not distinguish the occurrence of methylation at both cytosine residues and methylation at the external cytosine within the recognition site. This finding also agreed with the work on an elite rice hybrid as reported by Xiong *et al*. (1999).

CONCLUSIONS

MSAP technique with pooled DNA analysis was used to assess the patterns of cytosine methylation in six *Cycas* and six *Zamia* species. From 364 bands, there were two groups of sex-polymorphic bands. The first group comprised 0001, 0010, 0100, 0101, 1000 and 1010 patterns which could be regarded as DNA methylation polymorphism and also considered as nucleotide sequences differences. However, polymorphism occurred in the second group (0110, 0111, 1001, 1011, 1101 and 1110) was only from DNA methylation. These results indicated that DNA methylation affected the sex determination mechanism in cycads, but the exact role is still unknown. We are investigating further for more information on the specific region affected by DNA methylation that controls sex determination in these plants.

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