CHAPTER 5

RAPD STUDY

5.1 Introduction

The development of the polymerase chain reaction (PCR) had a significant impact in almost all areas of molecular biology (Saiki et al., 1988) and modifications of the basic procedure have allowed the development of numerous assays for detecting variation at the nucleotide level (Korner and Livak, 1989).

Over the years, detection of genetic diversity has progressed to assay molecular DNA variation. The molecular techniques are well suited to clarify problems of cryptic species. One of the most popular methods for the investigation of genetic diversity has recently been Random Amplified Polymorphic DNA (RAPD). With this technique, scientist can reveal a level of genetic variation useful for distinguishing populations and sometimes species, and typically with more variation data than isozyme (Swartz and Brunsfeld, 2002).

A genetic marker based on differences in DNA sequences between individuals generally detects more polymorphism than morphological and protein-based markers (Botstein et al. 1980, Tanksley et al. 1989). Compared to other methods for detecting DNA polymorphisms, this technique has the potential advantage for the investigation of rare plants. It is relatively inexpensive and technically straightforward for conducting experiments and does not requires prior knowledge of the genome (Wang et al., 2004). RAPD-PCR can be used to amplify certain segment of a genome by using a short arbitrary primer (Nilsson et al., 1998). However, care must be taken when using RAPD-PCR, as several factor have been reported to affect the reproducibility of the method (Hilton et al., 1997)

The *M. punctatum* complex is a variable species. The results from anatomical, morphological and numerical studies suggested that there are likely eight taxa in this complex. There is a need for more information and for an in-depth investigation to clarify the taxonomic status of the taxa in the complex, and to infer phylogenetic relationship in the *M. punctatum* complex and its related taxa. For this reason, a RAPD technique was used as an objective.

5.2 Material and Methods

5.2.1 Plant materials

Eight taxa of the *M. punctatum* complex were used for RAPD-PCR analyses. Forty one samples from 15 localities were collected (Table 5.1).

5.2.2 DNA Extraction

Fresh leaves were used for DNA extraction by a modified method of Doyle and Doyle (1987) which uses CTAB (Cetyltrimethylammonium bromide) method (Dellaporta, et al. 1983). The following procedure had been used in this study:

A: For preparation, set the microcentrifuge tube filled with 500 µl of modified CTAB extaction buffer and, preheat CTAB buffer in water-bath to 65 °C

1. Add liquid nitrogen to a pestle containing 0.1g of leaf material and grind to powder with a mortar. After that, transfer powder to extraction buffer in a microcentrifuge tube, vertex 10 second, and incubate at 65 °C for 20-30 minutes, mixing every 10 minutes.
2. Add 500 µl of chloroform-isoamylalcohol mixture, invert gently 5 times and incubate by gently shaking at room temperature. Centrifuge at 10,000 rpm at 4 °C for 10 minutes.
3. Transfer the supernatant into a new microcentrifuge tube. Add 0.1 volume of 3M Sodium acetate, mix, then add 0.6 volume iced-cold (-20 °C) isopropanol, mix by inverting.
4. Let the tube stand at -20 °C for 30 minutes and centrifuge at 10,000 rpm at 4 °C for 10 minutes. Gently discard the supernatant (be careful not to disturb DNA pellet).
5. Add 500 µl of ice-cold 75% ethanol, gently invert the tube several times. Centrifuge at 10,000 rpm for 5 minutes, discard the supernatant and allow the pellet to air-dry.
6. Dissolve DNA in 200 µl of sterile TE buffer and store then DNA solution at -20 °C until use.

B: To increase the purity of DNA sample, optional steps for some samples are listed below:

1. Add 1 µl of RNAase (20 µg/ml in concentration) to digest RNA and incubate the solution at 37 °C for 30-60 minutes.
2. Add 200 µl of Phenol:Chloroform mixture, invert gently. Centrifuge at 10,000 rpm at 4 °C for 10 minutes.
3. Add 500 µl of Chloroform:Isoamylalcohol mixture, invert gently. Centrifuge at 10,000 rpm at 4 °C for 10 minutes. Then transfer supernatant into a new microcentrifuge tube.
4. Add 0.1 volume of 3M sodium Acetate, mix, then, add 2 volume iced-cold (-20 °C) absolute ethanol, invert gently and let the tube stand at -20 °C for 30 minutes. Centrifuge at 10,000 rpm at 4 °C for 10 minutes. Gently discard the supernatant (be careful not to disturb DNA pellet)
5. Add 500 µl of iced-cold 75 % ethanol, gently invert the tube several times and allow the pellet to air-dry.
6. Dissolve the DNA in 50-100 µl of sterile TE buffer and store DNA solution at -20 °C until use.

5.2.3 RAPD Analysis

Genetic variation at the molecular level among eight taxa of the *M. punctatum* complex and outgroup was detected by RAPD markers. The materials used in this analysis are shown in Table 5.1.

Screening of primers

Forty four primers of arbitrary ten-oligonucleotide sequences (Operon Technologies, Alameda and Genset Oligos) were screened for selecting appropriate primers which gave polymorphic, scorable, reproducible DNA product (bands).

Table 5.1 Sampling sites and numbers of DNA samples of *M. punctatum* complex and outgroup taxa used in RAPD analysis.

Species	Locality	No. of Individual	Abbre-viations
M. whiteheadii	1. Sumatra, Indonesia	6	W1-6
M. siamense	1. Bannang Sata District, Yala, Thailand	4	S1-4
M. thailandicum	1. Chumphon, Thailand	5	T1-5
	2. Na Yong District, Trang, Thailand	1	T6
M. membranaceum	1. Doi Inthanon National Park, Chiang Mai, Thailand	1	B1
	2. Suan Phueng District, Ratchaburi, Thailand	1	B2
M. musifolium	1. Waeng District, Naratiwat, Thailand	6	M1-6
M. punctatum	1. Khao Chong Waterfall, Trang, Thailand	2	P1-2
	2. Khoa Nan Peak, Nakhon Si Thammarat, Thailand	2	P3-4
	3. Na Yong District, Trang, Thailand	2	P5-6
	4. Mindanoa, Phillipines	4	PP1-4
	5. Suan Phueng District, Ratchaburi, Thailand	1	P7
	6. Ton Prae Tong Waterfall, Phatthalung, Thailand	1	P8
P. irioides	1.Ton Prae Tong Waterfall, Phatthalung, Thailand	3	I1-3
	3. Khao Chong Waterfall, Trang, Thailand	3	I4-6
M. punctatum cv. *grandicep*	1. Suan Luang District, Bangkok, Thailand	3	G1-3
	2. Kan Na Yao District, Bangkok, Thailand	2	G4-5
M. punctatum cv. *serratum*	1. Pathum Wan District, Bangkok, Thailand	2	E1-2
	2. Kan Na Yao District, Bangkok, Thailand	3	E3-5
Total		41	

RAPD-PCR condition and electrophoresis

Amplification was carried out in a 20 µl volume in 0.2 ml PCR microtube using a PTC-100 Peltier Thermal cycle. The reaction mixture contained 1.25 mM of dNTPs, 10x Taq DNA polymerase buffer (QIAGEN, Leusden, Netherlands), 50 mM $MgCl_2$, 2.5 picomole primer, 5 ng DNA, and 1 unit of Taq DNA Polymerase (QIAGEN, Leusden, Netherlands). The amplification cycle was performed as follows: initial 3 minutes at 94°C denaturation; 40 cycles of 0.1 minutes at 94°C, 0.2 minutes at 38°C annealing, 1 minutes at 72 °C; and 7 minutes at 72 °C extension. The amplified products were subsequently run on gel or stored at 4 °C overnight before electrophoresis.

Amplified fragments were separated in 1 % agarose gel using 1x TAE buffer and were visualized and photographed using a gel documentation analysis set (BIO RAD), after staining with Ethidium bromide.

Table 5.2 Twenty one RAPD primers, primer sequences and numbers of amplified loci per primer.

Primer Code	Nucleotide sequence	No. of amplified loci
OPS-1	5′ CTACTGCGCT 3′	9
OPS-2	5′ CCTCTGACTG 3′	10
OPS-7	5′ TCCGATGCTG 3′	10
OPS-9	5′ TCCTGGTCCC 3′	12
OPS-10	5′ ACCGTTCCAG 3′	7
OPS-11	5′ AGTCGGGTGG 3′	10
OPS-12	5′ CTGGGTGAGT 3′	12
OPS-14	5′ AAAGGGGTCC 3′	7
OPS-16	5′ AGGGGGTTCC 3′	6
OPS-17	5′ TGGGGACCAC 3′	12
OPS-18	5′ CTGGCGAACT 3′	11
OPS-20	5′ TCTGGACGGA 3′	15
UBC-718	5′ GGGAGAGGGA 3′	10
UBC-722	5′ CCTCTCCCTC 3′	19
UBC-729	5′ CCCAACCCAC 3′	13
UBC-731	5′ CCCACACCAC 3′	15
UBC-734	5′ GGAGAGGGAG 3′	10
UBC-742	5′ CCTCCCTCCT 3′	14
UBC-785	5′ CACCCAACCA 3′	13
GEN-867	5′ CCTGACTCTC 3′	18
Total		233

5.2.4 Data Analysis

Only clearly appearing bands of RAPD products were scored for presence. The presence or absence of bands was coded as binary (1, 0) data respectively. A SIMQUAL module which computes genetic similarity coefficients for qualitative data was calculated according to Dice (1945). The similarity matrix obtained was employed to find clusters by the Unweighted Pair Group Method using Arithmetic Averages (UPGMA), using the SAHN-clustering module, and a dendrogram was produced using the TREE programs all in NTSYS-pc, version 2.10m (Rohlf, 2000).

5.3 Results

5.3.1 RAPD Analysis

RAPD profile

Only 20 of 44 RAPD primers (Table 5.2) of arbitrary ten-oligonucleotide sequence screened were selected and used to analyses the genomic DNAs of 41 individual plants from various localities of *M. punctatum* complex (Table 5.2). These 20 primers generated 233 clear and reproducible bands (RAPD loci), varying in size from 0.25 to 3 kb. The number of bands per primer ranged from 6 (in OPS-16) to 19 (in UBC-722) with an average of 11.65 bands/primer. The number of polymorphic RAPD loci per species ranged from 1 to 12 (Table 5.3). Polymorphism in RAPD banding patterns among *M. punctatum* complex and outgroup was detected. The number of polymorphic RAPD loci per species ranged from 1 to 12 and given the percentage of polymorphic loci as 36.33-57.51 %(Table 5.3). The result also showed that there were no or only a slight polymorphism among populations of the same species.

Of all 233 different RAPD loci, 114-119 (48.93-51.07%) of *M. whiteheadii*, 104-108 (44.64-46.35%) of *M. siamense*, 115-122(49.36-52.36%) of *M. thailandicum*, 116-117(49.79-50.21%) of *M. membranaceum*, 102-105(43.78-45.06%) of *M. musifolium* were polymorphic. The percentage of polymorphic loci of *M. punctatum*, *M. punctatum* from Philippines, *M. punctatum* cv. *grandiceps*, and *M. punctatum* cv. *serratum* is 119-124(51.07-53.22%), 131-134(36.33-57.51%), 107-117(45.92-50.21%) and 112-121 (48.07-51.93%), respectively. Finally, in *P. irioides* 118-125 bands (50.64-53.65%) were detected (Table 5.3).

Table 5.3 Number and percentage of DNA polymorphism in the *M. punctatum* complex.

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
OPS-1	9	*M. whiteheadii*	8	88.89
		M. siamense	8	88.89
		M. thailandicum	5	55.56
		M. membranaceum	5	55.56
		M. musifolium	7	77.78
		M. punctatum	5	55.56
		P. irioides	6	66.67
		M. punctatum from Philippines	6	66.67
		M. punctatum cv. *grandicaps*	6	66.67
		M. punctatum cv. *serratum*	5	55.56
OPS-2	10	*M. whiteheadii*	8	80.00
		M. siamense	9	90.00
		M. thailandicum	9	90.00
		M. membranaceum	8	80.00
		M. musifolium	8-9	80.00-90.00
		M. punctatum	9-10	90.00-100.00
		P. irioides	9-10	90.00-100.00
		M. punctatum from Philippines	8	80.00
		M. punctatum cv. *grandicaps*	8	80.00
		M. punctatum cv. *serratum*	8	80.00
OPS-7	10	*M. whiteheadii*	4	40.00
		M. siamense	1	10.00
		M. thailandicum	2	20.00
		M. membranaceum	2-3	20.00-30.00
		M. musifolium	2-3	20.00-30.00
		M. punctatum	2	20.00
		P. irioides	2-3	20.00-30.00
		M. punctatum from Philippines	5	50.00
		M. punctatum cv. *grandicaps*	4-5	40.00-50.00
		M. punctatum cv. *serratum*	2-3	20.00-30.00
OPS-9	12	*M. whiteheadii*	2	16.67
		M. siamense	4-5	33.33-41.67
		M. thailandicum	5	41.56
		M. membranaceum	1	8.33
		M. musifolium	2	16.67
		M. punctatum	1	8.33
		P. irioides	4-5	33.33-41.67
		M. punctatum from Philippines	4	33.33
		M. punctatum cv. *grandicaps*	5-7	41.56-58.33
		M. punctatum cv. *serratum*	6-7	50.00-58.33

Table 5.3 (continued)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
OPS-10	7	*M. whiteheadii*	6-7	85.71-100.00
		M. siamense	6	85.71
		M. thailandicum	7	100.00
		M. membranaceum	6	85.71
		M. musifolium	5	71.42
		M. punctatum	6-7	85.71-100.00
		P. irioides	7	100.00
		M. punctatum from Philippines	6-7	85.71-100.00
		M. punctatum cv. *grandicaps*	5	71.42
		M. punctatum cv. *serratum*	6	85.71
OPS-11	10	*M. whiteheadii*	5	50.00
		M. siamense	1	10.00
		M. thailandicum	4-5	40.00-50.00
		M. membranaceum	4	40.00
		M. musifolium	2	20.00
		M. punctatum	4	40.00
		P. irioides	5	50.00
		M. punctatum from Philippines	4-5	40.00-50.00
		M. punctatum cv. *grandicaps*	3-4	30.00-40.00
		M. punctatum cv. *serratum*	3-4	30.00-40.00
OPS-12	12	*M. whiteheadii*	5	41.67
		M. siamense	5-6	41.67-50.00
		M. thailandicum	2	16.67
		M. membranaceum	9	75.00
		M. musifolium	4	33.33
		M. punctatum	5-6	41.67-50.00
		P. irioides	5-6	41.67-50.00
		M. punctatum from Philippines	6	50.00
		M. punctatum cv. *grandicaps*	3-4	25.00-33.33
		M. punctatum cv. *serratum*	3-5	25.00-41.67
OPS-14	7	*M. whiteheadii*	2	28.57
		M. siamense	2	28.57
		M. thailandicum	4	57.14
		M. membranaceum	3	42.85
		M. musifolium	2	28.57
		M. punctatum	4	57.14
		P. irioides	3	42.85
		M. punctatum from Philippines	2	28.57
		M. punctatum cv. *grandicaps*	3	42.85
		M. punctatum cv. *serratum*	2-3	28.57-42.85

Table 5.3 (continued)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
OPS-16	6	*M. whiteheadii*	1	16.67
		M. siamense	1	16.67
		M. thailandicum	2	33.33
		M. membranaceum	2	33.33
		M. musifolium	2	33.33
		M. punctatum	3	50.00
		P. irioides	3	50.00
		M. punctatum from Philippines	3	50.00
		M. punctatum cv. *grandicaps*	3	50.00
		M. punctatum cv. *serratum*	2	33.33
OPS-17	12	*M. whiteheadii*	4	33.33
		M. siamense	4	33.33
		M. thailandicum	3	25.00
		M. membranaceum	3-4	25.00-33.33
		M. musifolium	3	25.00
		M. punctatum	4-5	33.33-41.67
		P. irioides	3	25.00
		M. punctatum from Philippines	5	41.67
		M. punctatum cv. *grandicaps*	3	25.00
		M. punctatum cv. *serratum*	3	25.00
OPS-18	11	*M. whiteheadii*	8-9	72.73-81.82
		M. siamense	9	81.82
		M. thailandicum	8	72.73
		M. membranaceum	8	72.73
		M. musifolium	9	81.82
		M. punctatum	8-9	72.73-81.82
		P. irioides	8-10	81.82-90.91
		M. punctatum from Philippines	8	72.73
		M. punctatum cv. *grandicaps*	8	72.73
		M. punctatum cv. *serratum*	8	72.73
OPS-20	15	*M. whiteheadii*	7	46.67
		M. siamense	5	33.33
		M. thailandicum	5	33.33
		M. membranaceum	3	20.00
		M. musifolium	3	20.00
		M. punctatum	6	40.00
		P. irioides	4-5	26.66-33.33
		M. punctatum from Philippines	9	60.00
		M. punctatum cv. *grandicaps*	10	66.67
		M. punctatum cv. *serratum*	8	53.33

Table 5.3 (continued)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
UBC-718	10	*M. whiteheadii*	3	30.00
		M. siamense	3	30.00
		M. thailandicum	3	30.00
		M. membranaceum	4	40.00
		M. musifolium	5	50.00
		M. punctatum	5	50.00
		P. irioides	5	50.00
		M. punctatum from Philippines	5	50.00
		M. punctatum cv. *grandicaps*	6-7	60.00-70.00
		M. punctatum cv. *serratum*	5-6	50.00-60.00
UBC-722	19	*M. whiteheadii*	7	36.84
		M. siamense	5	26.31
		M. thailandicum	4	21.05
		M. membranaceum	10	52.63
		M. musifolium	8	42.10
		M. punctatum	7	36.84
		P. irioides	5-6	26.32-31.58
		M. punctatum from Philippines	9	47.37
		M. punctatum cv. *grandicaps*	4	21.05
		M. punctatum cv. *serratum*	7-8	36.84-42.10
UBC-729	13	*M. whiteheadii*	6	46.15
		M. siamense	7	53.85
		M. thailandicum	11	84.62
		M. membranaceum	7	53.85
		M. musifolium	9	69.23
		M. punctatum	9	69.23
		P. irioides	8	61.54
		M. punctatum from Philippines	8	61.54
		M. punctatum cv. *grandicaps*	6-8	46.15-61.54
		M. punctatum cv. *serratum*	8-9	61.54-69.23
UBC-731	15	*M. whiteheadii*	10	66.67
		M. siamense	10	66.67
		M. thailandicum	8	53.33
		M. membranaceum	10	66.67
		M. musifolium	10	66.67
		M. punctatum	12	80.00
		P. irioides	9-10	60.00-66.67
		M. punctatum from Philippines	7	46.67
		M. punctatum cv. *grandicaps*	6-9	40.00-60.00
		M. punctatum cv. *serratum*	8-10	53.33-66.67

Table 5.3 (continued)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
UBC-734	10	*M. whiteheadii*	5	50.00
		M. siamense	2	20.00
		M. thailandicum	5-6	50.00-60.00
		M. membranaceum	4	40.00
		M. musifolium	3	30.00
		M. punctatum	4	40.00
		P. irioides	4	40.00
		M. punctatum from Philippines	4	40.00
		M. punctatum cv. *grandicaps*	4	40.00
		M. punctatum cv. *serratum*	4	40.00
UBC-742	14	*M. whiteheadii*	10	71.43
		M. siamense	10	71.43
		M. thailandicum	9-11	64.29-78.57
		M. membranaceum	9	64.29
		M. musifolium	8	57.14
		M. punctatum	9	64.29
		P. irioides	7-8	50.00-57.14
		M. punctatum from Philippines	12	85.71
		M. punctatum cv. *grandicaps*	5-6	35.71-42.86
		M. punctatum cv. *serratum*	8-9	57.14-64.29
UBC-785	13	*M. whiteheadii*	10	75.92
		M. siamense	11	84.62
		M. thailandicum	11	84.62
		M. membranaceum	8	61.54
		M. musifolium	9	69.23
		M. punctatum	11	84.62
		P. irioides	11	84.62
		M. punctatum from Philippines	12	92.31
		M. punctatum cv. *grandicaps*	7	53.85
		M. punctatum cv. *serratum*	12	92.31
GEN-867	18	*M. whiteheadii*	3	16.67
		M. siamense	2	11.11
		M. thailandicum	9	50.00
		M. membranaceum	9	50.00
		M. musifolium	2	11.11
		M. punctatum	5	27.78
		P. irioides	6-7	33.33-38.89
		M. punctatum from Philippines	8	44.44
		M. punctatum cv. *grandicaps*	9	50.00
		M. punctatum cv. *serratum*	6	33.33

Table 5.3 (continued)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
Total	233	*M. whiteheadii*	114-119	48.93-51.07
		M. siamense	104-108	44.64-46.35
		M. thailandicum	115-122	49.36-52.36
		M. membranaceum	116-117	49.79-50.21
		M. musifolium	102-105	43.78-45.06
		M. punctatum	119-124	51.07-53.22
		P. irioides	118-125	50.64-53.65
		M. punctatum from Philippines	131-134	36.33-57.51
		M. punctatum cv. *grandicaps*	107-117	45.92-50.21
		M. punctatum cv. *serratum*	112-121	48.07-51.93

Phenetic Analysis of RAPD data

For cluster analysis a total 233 RAPD markers were included. The UPGMA dendrogram derived from Dice's (1945) similarity coefficient splitted the 41 specimens into six groups (Fig. 5.2) at 0.50 phenon level. Specimens classified as group 1 composed of *M. punctatum P. irioides M. punctatum* cv. *grandicaps* and *M. punctatum* cv. *serratum*. Specimens classified as group 2, 3, 4, 5, and 6 are *M. whiteheadii*, *M. siamense*, *M. membranaceum*, *M. thailandicum*, and *M. musifolium*, respectively.

The dendrogram demonstrates that two cultivars of *M. punctatum*, *M. punctatum* cv. *grandiceps* and *M. punctatum* cv. *serratum*, are distinct from *M. punctatum* and its synonyms though they have very closely relationship. Furthermore, *M. musifolium*, one of synonym of *M. punctatum* according to Nooteboom's system (1997), is clearly separated from *M. punctatum*. The taxon *M. membranaceum* which was pointed out that it might be a variety of *M. punctatum* by Nooteboom (1997), exhibited a genetic similarity with *M. siamese*, *M. whiteheadii* and *M. thailandicum*. Thus, *M. punctatum* and its synonym, included *M. musifolium* and *M. membranaceum*, is not a well defined group according to the RAPD data.

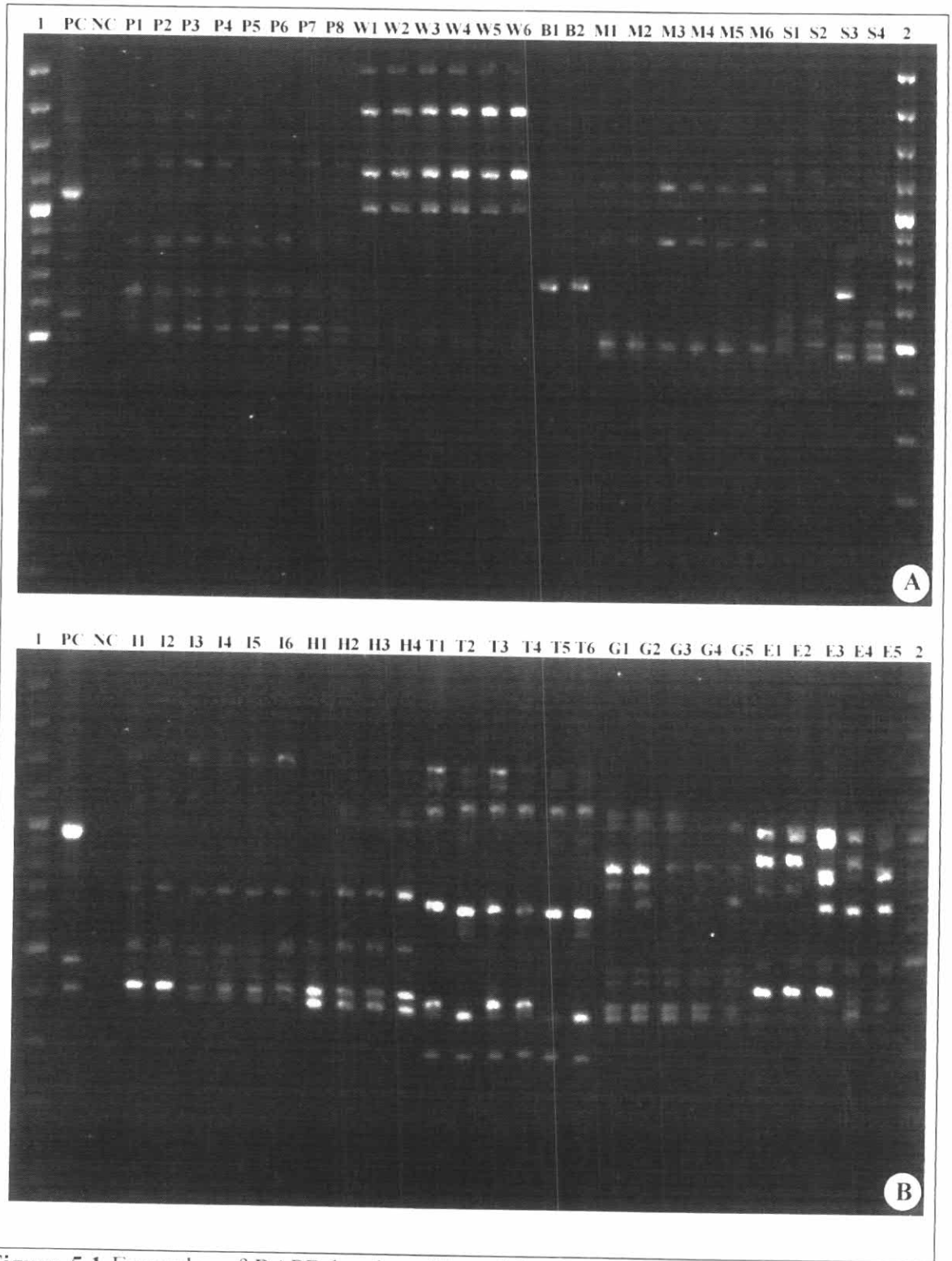

Figure 5.1 Examples of RAPD band profiles of the *M. punctatum* complex operated by primer OPS-20. Lane 1 and lane 2 (in A and B) are 1 kb DNA ladder. The abbreviation above each picture indicated populations of each species according to Table 5.1. The alphabet "PC" and "NC" is positive and negative control.

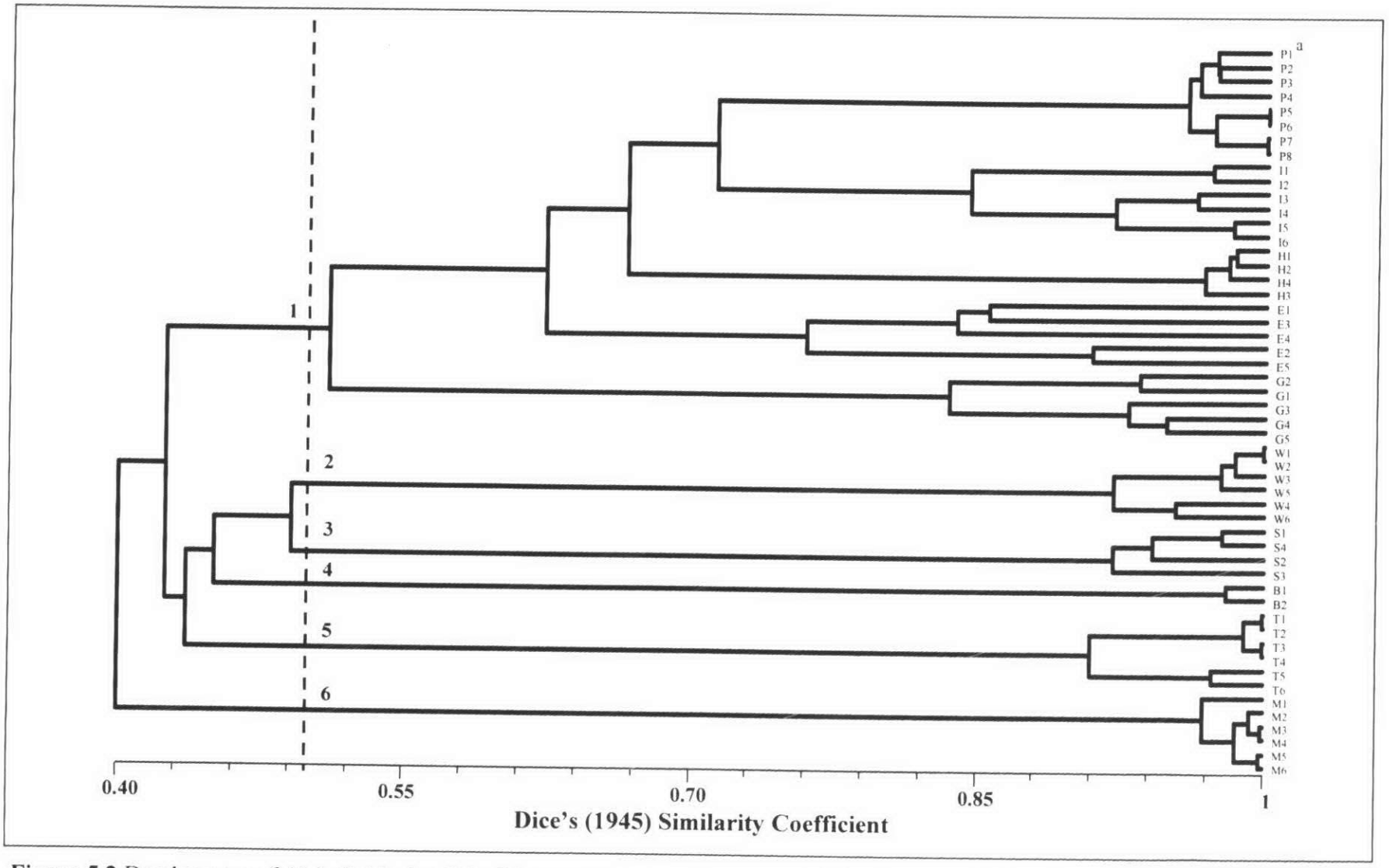

Figure 5.2 Dendrogram of 41 individuals of the *M. punctatum* complex generated by cluster analysis using UPGMA based on RAPD. (a. numbers correspond to those in Table 5.1).

5.4 Discussion and Conclusion

It can be seen (Fig. 4.1, 4.2 and 5.2) that both results from numerical and RAPD studies do not agree with the previous taxonomic classification of *M. punctatum* and its related taxa. Specimens classified as group 1 are similar to the result from cluster analysis of morphometric study.

Although *M. glossophyllum* and *M. steerei* were not included into this experiment due to technical limitation, conclusive results were obtained. The result from molecular analyses (RAPD-PCR) was congruent with both general morphological, anatomical and morphometric analyses. From UPGMA dendrogram derived from Dice's (1945) similarity coefficient in RAPD analysis, it can be seen that *M. punctatum* complex grouped into three groups viz. *M. punctatum* group, *M. thailandicum* group, and the last group comprised only one taxon, *M. musifolium*. The dendrogram also supported the genetically similarity between two locality of *M. punctatum*, from Thailand (P1-8) and Philippines (H1-4), and its synonym – *Polypodium irioides* (I1-6). The latter taxa were placed between *M. punctatum* from Thailand and Philippines (Fig 5.2). The result suggested that they are not significantly different genetically although from different locations. This finding corresponded with the results from the morphological study that pointed out that *P. irioides* is very similar to *M. punctatum* in many characters. It is reasonable to treat *P. irioides* as a synonym of *M. punctatum*.

Two cultivars of *M. punctatum*, *M. punctatum* cv. *grandiceps* (G1-5) and *M. punctatum* cv. *serratum* (E1-5), were placed in the same group with *M. punctatum*. It looks like *M. punctatum* cv. *serratum* have more genetic similarity with *M. punctatum* than *M. punctatum* cv. *grandiceps* (Fig 5.2). In addition, the morphological revealed that *M. punctatum* cv. *serratum* have same diagnostic characters with *M. punctatum* more than *M. punctatum* cv. *grandiceps*. Though these two varieties are close to *M. punctatum*, they still separate into independent subgroups. It thus makes sense to treat these two taxa as cultivars as in Nooteboom's taxonomic system.

Nooteboom published a classification system of microsoroid ferns in 1997. In this system, he included *M. musifolium* as synonym of *M. punctatum*. In contrast, the result from RAPD analysis agreed with both of the morphological and numerical studies that

separated *M. musifolium* from *M. punctatum*. This study did not support the previous classification of Nooteboom (1997) who proposed placing *M. musifolium* as synonym of *M. punctatum*. Here, *M. musifolium* is treated as a separate taxon following the classification system of Bosman (1991).

In the second group, *M. whiteheadii, M. siamense, M. membranaceum,* and *M. thailandicum*, displayed a close genetic relationships. They can be distinguished from each other at 0.54 of average taxonomic distance. It makes sense to propose these four taxa as independent species following the taxonomic classification system of Boonkerd (2006), Boonkerd, and Nooteboom (2001) and Smith and Hoshizaki (2000).

The characteristic of undulate frond margin, the diagnostic character to distinguish *P. irioides*, can be found in transplanted plants of *M. punctatum* preserved their anatomical, morphological and molecular characteristics in the greenhouse in dry season. The morphological difference due to stress was reported in many times (Tenhunen et al., 1985; Chen and Chen, 2005; Reudink et al. 2005; Masood and Abraham, 2006). So, it made sense to propose the hypothesis that this morphological difference might be due to stress.

The results from this study revealed that genetic variation can be detected in the same taxon that grows in different localities such as *M. punctatum* collected from Thailand and Philippines. The later plants have differed in some morphological character with the former such as fronds size and color. Frond of Philippines plants is thinner and dark green though reach sunlight in open areas, while *M. punctatum* collected from Thailand have thicker and light green fronds in same condition. This study and morphometric analyses indicated that *M. punctatum* collected in Thailand and Philippines are quite morphologically different from each other was supported by molecular data. It can be concluded that the morphological difference among taxa might come from the genetic variation (Paris, 1989; Li and Haufler, 1999).

Finally, according to RAPD data, the *M. punctatum* complex should be divided into independent taxa namely *M. whiteheadii*, *M. siamense*, *M. musifolium*, *M. membranaceum*, *M. thailandicum*, and *M. punctatum*; and also recognize two cultivars, *M. punctatum* cv. *serratum* and *M. punctatum* cv. *grandiceps*.