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Evaluation of Genetic Diversity of Wan Khanmak Herb Using AFLP Marker

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ABSTRACT

Wan Khanmak is a common name of Thai herb which is widely used as longevity enhancer. This medicinal species was previously classified as *Aglaonema simplex* (Blume) Blume. This experiment aims at collecting Wan Khanmak germplasms and evaluating the genetic diversity of this herb using DNA marker. The Amplified fragment length polymorphism (AFLP) technique was used to generate DNA fingerprint of Wan Khanmak samples which were collected throughout Thailand. When considering both DNA fingerprint data and morphological information, it revealed that a total of 115 Wan Khanmak accessions could be divided into seven major clusters. Cluster I consisted of 91 samples which were classified as *Aglaonema simplex* (Blume) Blume. The cluster II-VII were assigned to six species; *Aglaonema* sp. (1), *Aglaonema modestum* Schott ex Engl., *Aglaonema cochinchinense* Engl., *Aglaonema ovatum* Engl., *Aglaonema* sp. (2) and *Aglaonema nitidum* (Jack) Kunth, respectively. Genetic similarity between samples were calculated and the results ranged from 0.24-1.00 with an average of 0.627. The average of genetic similarity coefficient of *A. simplex* Bl. samples in cluster I was about 0.808, indicating that this species has low to moderate level of genetic variation. The *A. simplex* Bl. could be further divided into 12 subgroups. This experiment also indicated that there were other *Aglaonema* species that were misused as *A. simplex* Bl., because some *Aglaonema* spp. have similarities in general morphology and growth habitat.

Keywords: Wan Khanmak, *Aglaonema simplex*, herb, germplasm collection, DNA markers, AFLP

1. INTRODUCTION

Aglaonema simplex (Blume) Blume, generally known as 'Wan Khanmak', is herbaceous evergreen plant, a member of the family Araceae. It is widely distributed in humid and heavily shade forests of south-east Asia through north-eastern India and southern China [1-3]. *A. simplex* Bl. is an erect herb up to 120 cm tall; leaves obtuse, rounded or subtruncate at base, not variegated, margin of petiole usually with a membranous margin; peduncle 2-6 cm, fruiting ones up to 11 cm. Stalk of the spadix 0.5-1 cm, after anthesis slightly elongate; spadix erect, 2.5-4 cm. Fruit ellipsoid, obtuse, crowned by persistent stigma, ripe 1.5-1.75 cm, orange to red; pericarp thin; well-developed fruits in each

spadix 2 to many; staminodes with a roundish upper surface [4]. Recently, this herb is one of the commercially valuable medicinal plants. In Thai traditional medicine, Wan Khanmak fruit is extensively used as longevity enhancer and anti-asthma medication. This plant is believed to be a cure-all medicine. *A. simplex* Bl. crude extracts contained phytochemicals that plays a role in reducing atherosclerosis [5]. Pheophorbide-related compounds extracted from the leaves and stems of *A. simplex* Bl. are photosensitizers which have high potential for being used in photodynamic therapy to cure cancer [6]. However, the knowledge or scientific researches about this herb were still insufficient. Germplasm collection and genetic information about this herb are critical for other research fields such as conservation, pharmacology and breeding.

In recent years, DNA markers have proved to be the most effective methods for genetic diversity analysis. Among the various DNA marker systems, the amplified fragment length polymorphism (AFLP), developed by Vos *et al.* [7], is considered as a powerful DNA marker for revealing genetic polymorphisms with its informativeness, reproducibility and fewer reported reaction artifacts [8]. The previous knowledge of DNA sequences is not required for AFLP, so this technique is suitable for the first genetic variation study, where little or no preliminary data exist [9]. AFLP marker has been used in the assessment of genetic relationships of various plants species, including *Aglaonema*. Chen *et al.* [10] reported that AFLP fingerprint technique can be used to evaluate genetic relationships among ornamental *Aglaonema* species and cultivars which were conserved at Mid-Florida Research and Education Center.

This research project was setting up for collecting Wan Khanmak germplasms from all area throughout Thailand. Then, the genetic diversity of germplasms will be evaluated using DNA fingerprint technique. This experiment will provide an invaluable germplasm source. Moreover, the genetic variation and the variety identification results will be obtained. Both germplasm and genetic diversity information are very useful for other research fields such as pharmacology and toxicology, because the genotypic variation of herb might contain various levels of biologically active compounds. Therefore, this basic research is a prerequisite for further research in the future.

2. MATERIALS AND METHODS

2.1 Plant Materials and Genomic DNA Extraction

The herbs that generally called 'Wan Khanmak' were collected throughout Thailand. The germplasms from local markets were maintained at Division of Agricultural Science, Mahidol University, Kanchanaburi Campus, Kanchanaburi province, Thailand. As for the herbs which were found in conservative areas, such as National Reserved Forest and National park, their morphological characters were recorded. A young leaf was cut and then kept in zip-lock bag for DNA extraction.

Total genomic DNA was extracted from leaf tissue by using the 2x cetyltrimethylammonium bromide (CTAB) protocol [11]. The concentration of DNA was quantified by measuring the absorbance of UV light (260 nm) by spectrophotometer and then adjusting the concentration to 50 ng/μL for AFLP analysis.

2.2 AFLP Analysis

Genomic DNA (0.25 μg) was digested with 2.5 units of *Eco*RI and *Mse*I (Biolabs, Australia) in a final volume of 25 μL containing digestion reaction solution (50 mM potassium acetate, 20 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 1mM dithiothreitol, 0.1 mg/mL BSA). After mixing, the DNA samples were incubated for 3 h at 37ºC. Ligation of *Eco*RI and *Mse*I adaptors was performed by mixing 25 μL of double digested DNA and 25 μL of ligation solution (1 unit of T4 DNA ligase, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP). The mixture was then incubated at 25ºC for 2 h.

The pre-selective amplification reaction was performed using 2 μL of digestion/ligation reactions, in 25 μL of PCR reaction containing 200 mM Tris-HCl pH 8.4, 500 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pmol of *Eco*RI and *Mse*I adapter-directed primers (each possessing a single selective base, E+1; M+1) and 1 U of *Taq* DNA polymerase (Invitrogen, Brazil). PCR reactions were performed with the following profile: 94ºC for 3 min, 30 cycles of 30 s denaturing at 94ºC, 30 s annealing at 56ºC and 60 s extension at 72ºC, ending with 5 min at 72ºC to complete extension. After checking for the presence of a smear of fragments (100–1000 bp in length) by agarose gel electrophoresis, the amplification product was diluted 20 times in 0.1 × TE. Selective amplification (second PCR) of the diluted pre-amplification products was carried out using eight primer combinations (Table 2). Selective PCR reactions were performed with the following profile: 94ºC for 60 s, 36 cycles of 30 s denaturing at 94ºC, 30 s annealing and 60 s extension at 72ºC, ending with 10 min at 72ºC to complete extension. Annealing was initiated at a temperature of 65ºC, which was then reduced by 0.7ºC for the next 12 cycles and maintained at 56ºC 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 95ºC for 5 min and separated on 6% denaturing polyacrylamide gels (6% polyacrylamide 29:1, 7 M urea) in 1×TBE buffer. The gels were pre-run at 1500 V for about 30 min before 10 μL of the mix was loaded. Gels were run at 1500 V for about 3 h. The AFLP fragments were visualized by silver staining [12].

2.3 Data Analysis

For the diversity analysis, each PCR product was assumed to represent a single locus and only polymorphic bands were scored as present (1) or absent (0). A binary matrix was imported into NTSYS-pc version 2.20k for cluster analysis [13]. Genetic similarity among all accessions was calculated according to Jaccard's Similarity Index (JSI) [13] by the SIMQUAL subprogram, and the SAHN subprogram was used for cluster analysis by the UPGMA method (unweighted pair-group method with arithmetic means) [14]. A co-phenetic matrix was generated using the hierarchical cluster system, by means of the COPH routine, and correlated with the original distance matrices for the AFLP data, in order to test for agreement between the cluster in the dendrogram and the JSI matrix. The polymorphic information content (PIC), which is an index for the analysis of the polymorphism of each amplified DNA fragment, was calculated by Equation 1 [14] as following:

$$PIC = 1 - \sum_{i}^{k} Pi^2$$

where: Pi = allele frequency, i to k are alleles presented at each locus

3. Results and Discussion

In total, 115 accessions of Wan Khanmak were collected from both natural habitats and cultivated sites. The germplasms were conserved at Division of Agricultural Science, Mahidol University, Kanchanaburi Campus, Kanchanaburi. The accession numbers and location sites are shown in Table 1.

AFLP marker was employed to analyze the genetic diversity of Wan Khanmak germplasm collection. Genetic diversity was evaluated by eight AFLP selective-primer combinations. A total of 275 DNA bands was generated with an average of 34.38 bands per primer, while the range for the eight primer combinations were 17-65 (Table 2). The number of polymorphic bands was 270 (98.18% of the total bands) with an average of 33.75 bands per primer pair. The percentage of polymorphic bands in this study was higher than reported by Chen *et al.* [10]. The result obtained by Jaccard's coefficient showed that the genetic similarity varied from 0.157 to 1.00. The mean similarity was 0.723. The PIC value

Table 1. List of samples used in this study.

Accession no.	Collection site	Geographical area	Accession no.	Collection site	Geographical area
1	Ratchaburi La: 13.514628 Lo: 99.789531	West	61	Nakhon Ratchasima La: 14.6683595 Lo: 101.2107082	Northeast
2	Ratchaburi La: 13.514628 Lo: 99.789531	West	62	Nakhon Ratchasima La: 14.631683 Lo: 101.22158	Northeast
3	Ratchaburi La: 13.514628 Lo: 99.789531	West	64	Kanchanaburi La: 13.979390 Lo: 99.590581	West
4	Ratchaburi La: 13.514628 Lo: 99.789531	West	65	Nan La: 19.114793 Lo: 100.810218	North
5	Ratchaburi La: 13.514628 Lo: 99.789531	West	66	Kanchanaburi La: 14.3097975 Lo: 98.9907936	West
6	Ratchaburi La: 13.514628 Lo: 99.789531	West	67	Kanchanaburi La: 14.2984766 Lo: 98.8980359	West
7	Ratchaburi La: 13.514628 Lo: 99.789531	West	68	Uttaradit La: 17.643086 Lo: 100.114434	North
8	Ratchaburi La: 13.514628 Lo: 99.789531	West	69	Kanchanaburi La: 13.979390 Lo: 99.590581	West
9	Ratchaburi La: 13.514628 Lo: 99.789531	West	70	Nan La: 18.650545 Lo: 100.745721	North
10	Ratchaburi La: 13.514628 Lo: 99.789531	West	71	Kanchanaburi La: 13.980780 Lo: 99.600425	West
11	Ratchaburi La: 13.514628 Lo: 99.789531	West	72	Chachoengsao La: 13.609839 Lo: 101.297362	East
12	Ratchaburi La: 13.514628 Lo: 99.789531	West	73	Sa Kaeo La: 13.4138601 Lo: 102.3231256	East
13	Ratchaburi La: 13.514628 Lo: 99.789531	West	75	Nonthaburi La: 13.800316 Lo:100.551090	Central
14	Ratchaburi La: 13.514628 Lo: 99.789531	West	76	Bangkok La: 13.800316 Lo:100551090	Central
15	Ratchaburi La: 13.514628 Lo: 99.789531	West	77	Bangkok La: 13.800316 Lo: 100551090	Central
16	Ratchaburi La: 13.514628 Lo: 99.789531	West	78	Chiang Mai La: 18.6076516 Lo: 98.8846426	North
17	Ratchaburi La: 13.514628 Lo: 99.789531	West	79	Bangkok La: 13.800316 Lo: 100551090	Central

Table 1. List of samples used in this study. (Continued)

Accession no.	Collection site	Geographical area	Accession no.	Collection site	Geographical area
18	Ratchaburi La: 13.514628 Lo: 99.789531	West	80	Bangkok La: 13.800316 Lo: 100551090	Central
19	Ratchaburi La: 13.514628 Lo: 99.789531	West	81	Bangkok La: 13.800316 Lo:100551090	Central
20	Ratchaburi La: 13.514628 Lo: 99.789531	West	82	Prachuap Khiri Khan La: 12.703785 Lo: 99.953136	West
21	Ratchaburi La: 13.514628 Lo: 99.789531	West	83	Prachuap Khiri Khan La: 12.188905 Lo: 99.972124	West
22	Ratchaburi La: 13.514628 Lo: 99.789531	West	84	Kanchanaburi La:13.977052 Lo:99.647164	West
23	Ratchaburi La: 13.514628 Lo: 99.789531	West	85	Kanchanaburi La: 14.336347 Lo: 98.976685	West
24	Ratchaburi La: 13.514628 Lo: 99.789531	West	86	Kanchanaburi La:14.336347 Lo:98.976686	West
25	Ratchaburi La: 13.514628 Lo: 99.789531	West	87	Kanchanaburi La: 14.336347 Lo: 98.976686	West
26	Ratchaburi La: 13.514628 Lo: 99.789531	West	88	Chumphon La: 10.492845 Lo: 99.122842	South
27	Ratchaburi La: 13.514628 Lo: 99.789531	West	89	Phitsanulok La: 17.189661 Lo: 100.270290	Central
28	Ratchaburi La: 13.514628 Lo: 99.789531	West	90	Ubon Ratchathani La: 15.233546 Lo: 104.717613	Northeast
29	Bangkok La: 13.747108 Lo: 100.3497406	Central	91	Kanchanaburi La: 14.665785 Lo: 99.316800	West
30	Bangkok La: 13.747108 Lo: 100.3497406	Central	92	Sa Kaeo La: 13.4138601 Lo: 102.3231256	East
31	Bangkok La: 13.747108 Lo: 100.3497406	Central	93	Sa Kaeo La: 13.4138601 Lo: 102.3231256	East
32	Bangkok La: 13.800316 Lo: 100551090	Central	94	Kamphaeng Phet La: 16.393740 Lo: 99.521029	Central
33	Ayutthaya La: 14.19521 Lo: 100.32512	Central	96	Trang La: 7.641429 Lo: 99.623124	South
34	Bangkok La: 13.800316 Lo: 100551090	Central	97	Phang Nga La: 8.826275 Lo: 98.370644	South

Table 1. List of samples used in this study. (Continued)

Accession no.	Collection site	Geographical area	Accession no.	Collection site	Geographical area
35	Lopburi La: 14.865876 Lo: 100.639195	Central	98	Phang Nga La: 8.826275 Lo: 98.370644	South
36	Lopburi La: 14.865876 Lo: 100.639195	Central	99	Ranong La: 10.478721 Lo: 98.803501	South
37	Prachuap Khiri Khan La: 12.308762 Lo: 99.921812	West	100	Trang La: 7.641429 Lo: 99.623124	South
38	Kanchanaburi La: 13.979390 Lo: 99.590581	West	101	Trang La: 7.641429 Lo: 99.623124	South
39	Surin La: 14.605744 Lo: 103.416501	Northeast	102	Trang La: 7.641429 Lo: 99.623124	South
40	Surin La: 14.605744 Lo: 103.416501	Northeast	103	Suphan Buri La: 14.786305 Lo: 99.352271	Central
41	Ubon Ratchathani La: 15.233546 Lo: 104.717613	Northeast	104	Suphan Buri La: 14.876284 Lo: 99.568671	Central
43	Ubon Ratchathani La: 15.233546 Lo: 104.717613	Northeast	105	Kanchanaburi La: 14.6670758 Lo: 99.3210673	West
45	Ubon Ratchathani La: 12.662743 Lo: 102.081825	Northeast	106	Kanchanaburi La: 14.070899 Lo: 98.996195	West
46	Sa Kaeo La: 12.662743 Lo: 102.081825	East	107	Suphan Buri La: 14.876284 Lo: 99.568671	Central
47	Chanthaburi La: 12.781153 Lo: 101.827311	East	108	Prachinburi La: 14.2213315 Lo: 101.4120555	East
48	Chanthaburi La: 12.781153 Lo: 101.827311	East	109	Prachinburi La: 14.2213315 Lo: 101.4120555	East
49	Chanthaburi La: 12.781153 Lo: 101.827311	East	110	Ranong La: 9.8563829 Lo: 98.625405	South
50	Chanthaburi La: 12.781153 Lo: 101.827311	East	111	Lampang La: 18.834659 Lo: 99.470408	North
51	Chanthaburi La: 12.662743 Lo: 102.081825	East	112	Lampang La: 18.834659 Lo: 99.470408	North
52	Chanthaburi La: 12.662743 Lo: 102.081825	East	113	Nakhon Si Thammarat La: 9.862889 Lo: 99.626889	South

Table 1. List of samples used in this study. (Continued)

Accession no.	Collection site	Geographical area	Accession no.	Collection site	Geographical area
53	Chanthaburi La: 12.662743 Lo: 102.081825	East	114	Surat Thani La: 8.915424 Lo: 98.528282	South
54	Ranong La: 10.478721 Lo: 98.803501	South	115	Trat La: 12.065356 Lo: 102.313300	East
55	Chonburi La: 13.3675676 Lo: 100.9903599	East	116	Trat La: 12.618491 Lo: 102.577127	East
56	Chiang Mai La: 18.807475 lo: 98.997271	North	117	Trat La :12.380833 Lo: 102.459125	East
57	Nakhon Ratchasima La: 14.631683 Lo: 101.221508	Northeast	118	Sa Kaeo La :14.151339 Lo: 102.661204	East
58	Nakhon Ratchasima La: 14.4355468 Lo: 101.4121754	Northeast	119	Nakhon Ratchasima La :14.287024 Lo:101.393667	Northeast
59	Nakhon Ratchasima La: 14.4355468 Lo: 101.4121754	Northeast	120	Sakon Nakhon La :17.123413 Lo:104.018708	Northeast
60	Nakhon Ratchasima La: 14.6683595 Lo: 101.2107082	Northeast			

Table 2. Average number of bands, number of alleles and proportion of polymorphic bands obtained for the 115 accessions from eight AFLP selective primer combinations.

Primer combination	No. of band	No. of polymorphic band	% polymorphic band	Average PIC
E[a]-AAG/M[b]-CTG	42	42	100	0.17
E-AGC/M-CTG	39	38	96.88	0.19
E-ACT/M-CTA	28	27	96.88	0.11
E-AAG/M-CTA	65	65	100	0.16
E-AGC/M-CTC	22	22	100	0.38
E-AAG/M-CAA	30	30	100	0.20
E-AGT/M-CAA	32	31	96.88	0.22
E-AGC/M-CAA	17	15	88.24	0.20
Total	275	270	-	-
Average	34.38	33.75	98.18	0.20

E [a] = pre-amplification primer (5'-GACTGCGTACCAATTC-3') of *Eco*RI
M [b] = pre-amplification primer (5'-GATGAGTCCTGAGTAA-3') of *Mse*I

ranged from 0.11-0.32 (mean = 0.20), suggested low to moderate genetic variation. This PIC value indicated the efficiency of using AFLP markers for investigating genetic relationships of *Aglaonema* species.

UPGMA analysis of genetic similarity estimates was performed using NTSYS version 2.20k. The co-phenetic correlation coefficient (r-value) between the AFLP-based data dendrogram and the similarity matrix clustering was 0.99, demonstrating a good fit between the dendrogram clusters and the similarity matrix from which they were derived. The dendrogram based on the UPGMA method revealed that all samples could be divided into seven major clusters at a cut-off genetic similarity value of about 0.582 (Figure 1). The members of each cluster were shown in Table 3. The morphological traits of the plants in each cluster were recorded (data not shown). The plant species were identified by taxonomic keys [1-3].

The morphological characteristics of samples in each cluster were shown in Figure 2 and plant morphology was described in Table 4. Cluster I consisted of 91 samples which were classified as *Aglaonema simplex* (Blume) Blume. The cluster II -VII were assigned to six species; *Aglaonema* sp. (1), *Aglaonema modestum* Schott ex Engl., *Aglaonema cochinchinense* Engl., *Aglaonema ovatum* Engl., *Aglaonema* sp. (2) and *Aglaonema nitidum* (Jack) Kunth, respectively (Table 3-4). However, the samples in cluster II and VI could not be identified at the species level, because their morphological characteristics were ambiguous when compared to the previous taxonomic reference data [1-3]. *Aglaonema* sp. (1) morphology was very similar to *A. simplex* but its leaf blade was obviously thinner. In another case, leaf base of *Aglaonema* sp. (2) was cordate, which was distinct from obtuse to rounded in *A. cochinchinense* (Table 4).

Cluster II contained only one sample, accession No.111 which was found in Chae Son National Park, Lampang province. An accession No.114, a lone member of cluster VI, was obtained from Khao Sok national park, Surat Thani province. Their morphological characteristics were slightly different from the reference taxonomic keys. Since samples in cluster II and VI coexisted with other *Aglaonema* species in the forests and *Aglaonema* possess unisexual, dichogamous flowers which promotes outcrossing, those unidentified samples might be the results of isolated habitat, natural interspecific hybridization or could be considered as new-found species. These hypotheses were supported by Jaccard's genetic similarity coefficients derived from AFLP analysis. Chen *et al.* [10] used AFLP to evaluate the genetic relationship among ornamental *Aglaonema* species and cultivars which were conserved at Mid-Florida Research and Education Center. The Jaccard's similarity coefficients among different species of *A. commutatum, A. crispum, A. modestum, A. nitidum and A. pictum* ranged from 0.31-0.79. As shown in Figure 1, the samples in cluster I and II were separated at the genetic similarity of about 0.48, while the successfully identified species which most of members of each cluster have similarity coefficient above 0.7. Thus, cluster I and II in our experiment might be attributed to two different species. To confirm this hypothesis, further studies with cytological analyses should be performed to investigate the progenitors of these two unidentified accessions. Since the hybrid contains two or more sets of chromosomes from different species, chromosome count combines with fertility study will be able to shed the light whether the plants were actually be hybrid in origin or not.

A dendrogram in the present study also showed that the members of five identified species were clearly separated. *A. nitidum* samples in cluster VII were found to be the most diverged germplasm. The mean values of genetic similarity coefficients among seven clusters were shown in Table 5. *Aglaonema* sp. (1) in cluster II and *Aglaonema* sp. (2) in cluster VI showed the most dissimilar since their Jaccard's genetic similarity coefficients is only 0.1610, while *Aglaonema cochinchinense* and

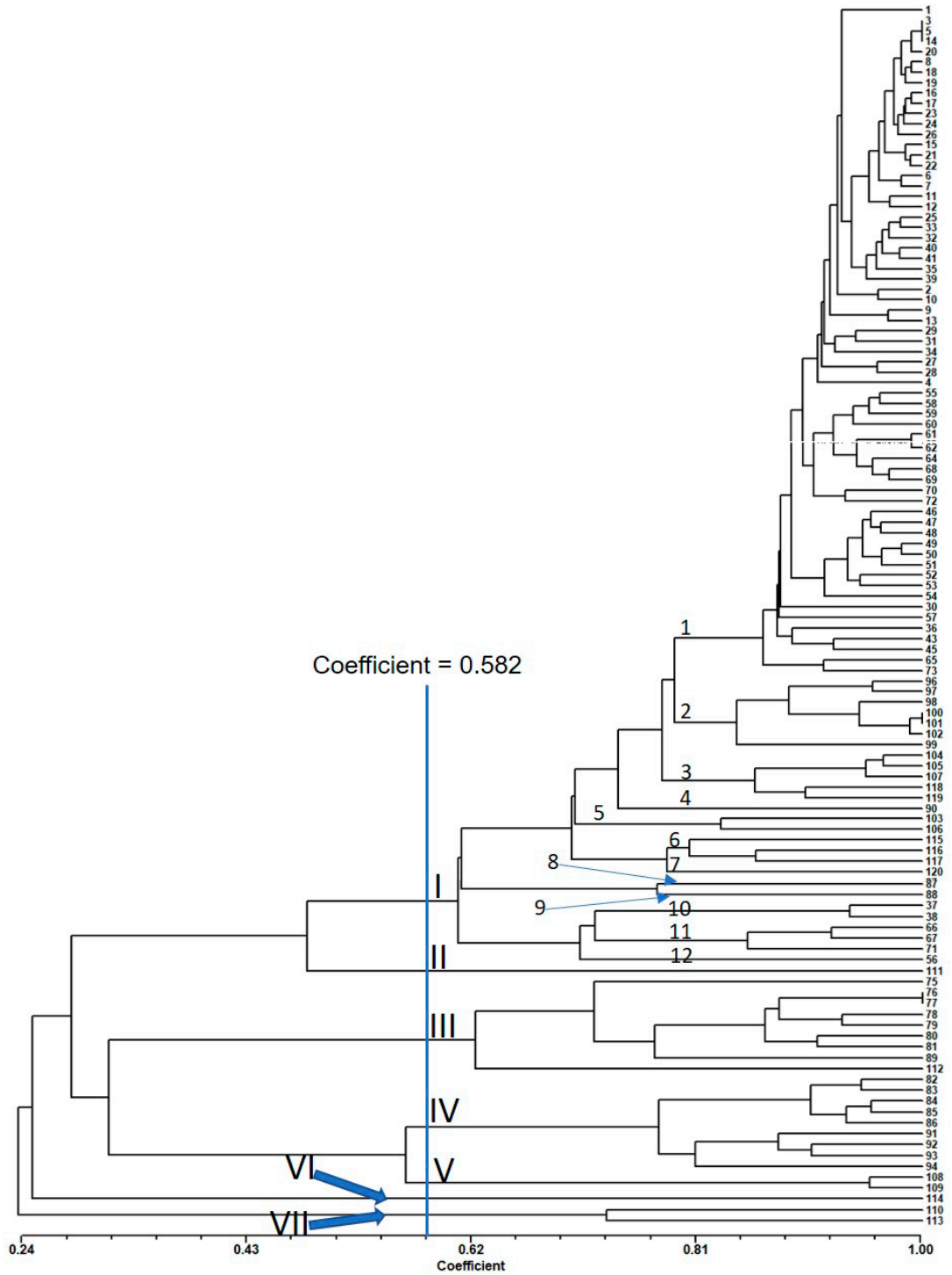

Figure 1. UPGMA clustering of 115 Wan Khanmak samples based on AFLP polymorphisms.

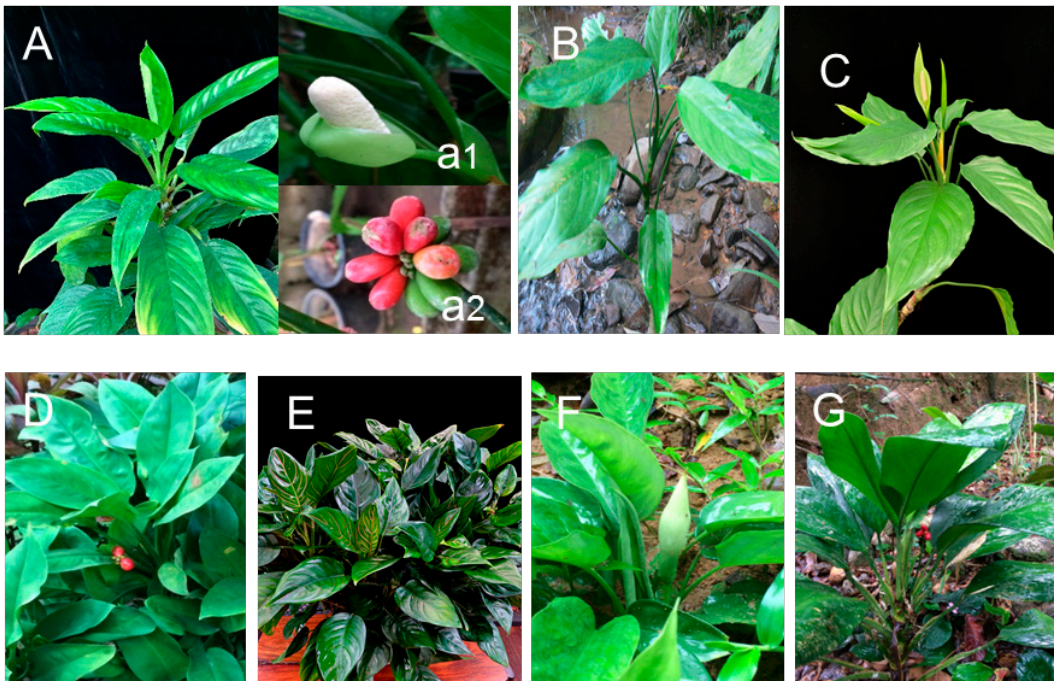

Figure 2. Plant morphology of *Aglaonema* species; (A) *A. simplex*, (a1) *A. simplex* inflorescence, (a2) *A. simplex* fruits, (B) *Aglaonema* sp. (1), (C) *A. modestum* Schott ex Engl., (D) *A. cochinchinense* Engl., (E) *A. ovatum* Engl., (F) *Aglaonema* sp. (2) and (G) *A. nitidum* (Jack) Kunth.

Table 3. Samples in each cluster.

Cluster	Accession number	No. of sample
I	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 43, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 87, 88, 90, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 115, 116, 117, 118, 119 and 120	91
II	111	1
III	75, 76, 77, 78, 79, 80, 81, 89 and 112	9
IV	82, 83, 84, 85, 86, 91, 92, 93 and 94	9
V	108 and 109	2
VI	114	1
VII	110 and 113	2
Total		115

Aglaonema ovatum are the most related species among the seven clusters with the average genetic similarity coefficients of 0.5669.

Furthermore, we found that there were other *Aglaonema* species that were misused as *A. simplex* Bl., especially *Aglaonema modestum*, because some *Aglaonema* spp. have similarities in general morphology and growth habitat (Figure 2). Misidentification of the herbal plant can lead to its substitution with potentially toxic plants. For example, *Curcuma comosa*, locally called 'Wan chak modlook', has been used generally in Thai

Table 4. Morphological characteristics of samples in each cluster.

Cluster	Species	Morphological characteristics
I	*Aglaonema simplex* (Blume) Blume.	Medium, slender to somewhat robust, sub-pachycaul to suffruticose or decumbent, evergreen herbs. *Stem* erect, 15–120 cm tall, 0.4–1.7(–2.5) cm thick. *Leaves* several to rather many together, often clustered towards the shoot tips in larger plants; *petioles* 4.3–21.5 cm long; *petiolar sheath* with a membranous margin; *leaf blade* narrowly oblong, narrowly elliptic to lanceolate, occasionally linear, elliptic or ovate, 10–35 by 1.9–25 cm, base often unequal or oblique, obtuse, rounded or subtruncate, rarely acute or subcordate; apex often apiculate, acuminate, sometimes abruptly or gradually acuminate; *primary lateral veins* strongly differentiated, 3–14 per side; *interprimary veins* much less prominent, blade plain green, never variegated but the shade of green varying markedly between populations, thinly leathery. *Inflorescences* 1–6 together; *peduncle* 4–12 cm; *spathe* oblong-ovate, often apiculate, inflating at pistillate anthesis, then soon caducous at onset of staminate anthesis, 1.8–6.5 cm, decurrent for 3–15 mm, white; *spadix* cylindrical, equalling or slightly exceeding spathe, 1.7–4.3 cm, stipitate; *stipe* 2–12 mm; *pistillate flower zone* 3–10 mm long, with 12–38 flowers; *staminate flower zone* 1.5–3.8 by 0.5 cm, white, separated from the pistillate zone by a brief naked interstice. *Fruits* ovoid-ellipsoid, 1–1.7 by 0.8 cm, green, ripening through yellow to bright red.
II	*Aglaonema sp.* (1)	Medium, slender to somewhat robust, sub-pachycaul to suffruticose or decumbent, evergreen herbs. *Stem* erect, 100 cm tall, 1.0–1.2 cm thick. *Leaves* several to rather many together, often clustered towards the shoot tips, almost the same as leaf blade of *A. simplex* but thinner; *petioles* 4.3–21.5 cm long; *petiolar sheath* with a membranous margin; *leaf blade* narrowly oblong, narrowly elliptic to lanceolate, occasionally linear, elliptic or ovate, 35 by 20 cm, base unequal or oblique, obtuse, rounded or subtruncate; apex acuminate; *primary lateral veins* strongly differentiated, 3–14 per side; *interprimary veins* much less prominent, blade plain green, never variegated,. *Inflorescences* not found, cannot collected the data. *Fruits* ovoid-ellipsoid, 1–1.7 by 0.8 cm, green, ripening through yellow to bright red.
III	*Aglaonema modestum* Schott ex Engl.	Small to medium, erect to eventually decumbent, evergreen herb. *Stem* erect, 20–50 cm tall, 0.4–2 cm thick. *Leaves* few to several together; *petioles* 6–22 cm long, upper portion flattened; *petiolar sheath* broad, membranous, 2.5–11 cm long; *leaf blade* ovate to sublanceolate, rarely elliptic, 12–25 by 5–11 cm, base unequal, obtuse to rounded, apex gradually long acuminate; *primary lateral veins* 4–6 diverging from the midrib at 30°–50°; *interprimary veins* much less prominent. *Inflorescence* 1–3 together; *peduncle* 5–12.5 cm; *spathe* elongate-oblong, apiculate, 3–9 by 1.5–2.6 cm, opening pale green and ageing to yellowish, decurrent for 0.5–2 cm; *spadix* thin-cylindrical, sessile, 1.5–4.3 cm long, ca 0.6 –3.1 cm short of spathe apex; *pistillate flower zone* 0.5–1 cm long, adnate to spathe, with 9–13 flowers; *staminate flower zone* 2.3–3.5 by 0.3–0.6 cm, white. *Fruits* ellipsoid, 2–3 by 1–1.3 cm, green, ripening to dark orange.

Table 4. Morphological characteristics of samples in each cluster. (Continued)

Cluster	Species	Morphological characteristics
IV	*Aglaonema cochinchinense* Engl.	Small, moderately robust, erect evergreen herb. *Stem* erect, not exceeding 15 cm. *Leaves* several together; *petioles* 8–22 cm; *petiolar sheath* short, with membranous margins, 3–5 cm long; *leaf blade* ovate to lanceolate to elliptic or narrowly elliptic, rather thinly leathery, 11–28 by 4–12.5 cm, base obtuse to rounded, frequently unequal, apex obtuse to acute, sometimes apiculate; mid-rib conspicuously raised adaxially; *venation* undifferentiated in living and dried plants. *Inflorescences* 1–2 together; *peduncle* 6–15 cm, frequently exceeding the subtending petiole; *spathe* oblong-ovate, 3–5 cm long, often apiculate, decurrent for 0.5–2 cm.; *spadix* cylindrical, 1.9–3.2 cm long, often exceeding the spathe at anthesis, stipitate; *stipe* 0.1–1 cm long; *pistillate flower zone* 0.4–0.7 cm long, with ca 10 flowers, these occasionally with clavate, associated staminodia; *staminate flower zone* tapering-cylindrical, 1.5–3 by ca 0.4 cm, white. *Fruits* ellipsoid, ca 2 by 1 cm, green, ripening to red.
V	*Aglaonema ovatum* Engl.	Small, erect to decumbent, evergreen herb, to 50 cm but usually much less. *Stem* erect to 50 cm, then decumbent and sprawling, 0.5–1.5 cm thick, decumbent stems often producing greatly thickened roots. *Leaves* few together; *petioles* 22 cm long; *petiolar sheath* membranous, 0.3–0.7 times as long as the petiole; *leaf blade* ovate to lanceolate, rarely elliptic, 10–26 by 3–10.5 cm, base rounded to obtuse, rarely subcordate; apex acuminate, apiculate, pale green; *primary lateral veins* 5–10; *interprimary veins* barely or only weakly differentiated. *Inflorescences* 1–2 together; *peduncle* 3.5–10 cm long, usually about ½ as long as the subtending petiole; *spathe* ovate, 3.5–4.7 cm long, decurrent for 0.4–1.5 cm, white, spreading at anthesis; *spadix* cylindrical, sessile, 3–4.3 cm long, usually exceeding spathe but sometimes 0.5 cm short of spathe apex; *pistillate flower zone* 0.4–1.6 cm, with ca 10–20 flowers; *staminate flower zone* 2.8–3.5 by 0.6–1 cm, white with lower 0.5 cm sterile and dark cream. *Fruits* ellipsoid, 1.1–2 by 0.7–1 cm, green, ripening through yellow to red.
VI	*Aglaonema sp.* (2)	Small, erect to decumbent, evergreen herb. *Stem* erect or with lower part reclining on ground in larger plants. *Leaves* several to rather many together, usually restricted to the terminal portion of stems in larger plants; *petioles* deeply channelled, 22 cm long; *petiolar sheath* membranous, 3.0–5.0 cm, margins scarious; *leaf blade* narrowly elliptic to narrowly oblong or oblanceolate, 11–50 by 4–20 cm, base cornate; *venation* barely or not differentiated. *Inflorescences* 2–5 together; *peduncle* 5–21 cm long, equalling or surpassing the subtending petiole; *spathe* oblong, 3–8.5 cm, decurrent for 4–20 mm, white at anthesis, becoming green during fruiting, persistent until fruit full-sized but still green, then marcescent; *spadix* cylindrical, equalling or slightly exceeding spathe, 1.3–7.0 cm, stipitate; *stipe* 0.2–0.9 cm long; *pistillate flower zone* 0.5–1 cm long with 16–37 flowers; *staminate flower zone* 1.1–6.0 by ca.1.5 cm, white. *Fruits* ellipsoid, green, changing to white then pink and finally red.

Table 4. Morphological characteristics of samples in each cluster. (Continued)

Cluster	Species	Morphological characteristics
VII	*Aglaonema nitidum* (Jack) Kunth	Medium to large, somewhat robust, pachycaul or decumbent, evergreen herb, to 2 m tall. *Stem* erect or with lower part reclining on ground in larger plants, 0.5–5 cm thick. *Leaves* several to rather many together, usually restricted to the terminal portion of stems in larger plants; *petioles* deeply channelled, 8-29 cm long; *petiolar sheath* extending to the petiole tip, margins scarious; *leaf blade* narrowly elliptic to narrowly oblong or oblanceolate, 11–50 by 4–20 cm, base cuneate to attenuate, rarely broadly acute, apex acuminate to broadly acute or shortly acuminate, apiculate, coriaceous, usually plain green, sometimes with grey variegation either in bars following the venation or in rather irregularly scattered blotches; *venation* barely or not differentiated, weakly differentiated into 5–9 or more. *Inflorescences* 2–5 together; *peduncle* 5–21 cm long, equalling or surpassing the subtending petiole; *spathe* oblong, 3.0–8.5 cm, decurrent for 4–20 mm, white at anthesis, becoming green during fruiting, persistent until fruit full-sized but still green, then marcescent; *spadix* cylindrical, equalling or slightly exceeding spathe, 1.3–7 cm, stipitate; *stipe* 0.2–0.9 cm long; *pistillate flower zone* 0.2–1.0 cm long with 16–37 flowers; *staminate flower zone* 1.1–6.0 by ca.1.5 cm, white. *Fruits* ellipsoid, green, changing to white then pink and finally red.

Table 5. The mean values of genetic similarity coefficients among seven clusters.

Sample clusters	I	II	III	IV	V	VI	VII
I	-	-	-	-	-	-	-
II	0.4839	-	-	-	-	-	-
III	0.2957	0.2096	-	-	-	-	-
IV	0.2891	0.1768	0.3237	-	-	-	-
V	0.2425	0.2031	0.3237	0.5669	-	-	-
VI	0.2688	0.1610	0.1966	0.1878	0.1865	-	-
VII	0.2455	0.2819	0.2373	0.2191	0.2271	0.2091	-

indigenous medicine. *C. comosa* rhizome contains phytoestrogens, which are plant derived, estrogenic-like compounds [17]. The rhizome of *C. latifolia* Rosc. is morphologically similar to *C. comosa* but has less estrogenic activity and is very toxic. There was a report that *C. latifolia* had been misused as a substitute for *C. comosa* [18]. We are concerned that the same situation might occur by using relative species as a substitute for *A. simplex* Bl. However, the pharmacological activities and toxicities of *A. simplex* Bl. and related species have not yet undergone examination. Nevertheless, the AFLP DNA fingerprint data showed that *A. simplex* Bl. samples could be subdivided into 12 subgroups. These different genotypes might contain various levels of biologically active compounds. Further research should focus on the pharmacological and toxicological characterization of the related species and the various genotypes of *A. simplex* Bl. It may be possible to find a correlation between the plant species or genotypes and the variation in the biologically active compounds [19].

When focusing on *A. simplex* Bl. accessions which were grouped together in cluster I, Jaccard's similarity coefficient between samples in this group ranged from 0.61-1.00 with an average of 0.81. A

moderate to low level of genetic similarity among germplasm was found. This result might be caused by the high outcrossing rate. The heterozygous offspring derived from two distinctive progenitors increases genetic diversity. On the contrary, the genetic similarity between samples assessed by AFLP marker revealed that there are duplicate accessions in the germplasm collection studied. This outcome may happen because *A. simplex* Bl. can be clonal propagated by cutting, such as accession no.3, 5 and 14 which were collected from the same site.

All 91 *A. simplex* Bl. samples were further divided into 12 subgroups at the genetic similarity of 0.80 (Figure 1). Subgroup 1 is the largest group comprising 64 samples, 28 of which are from Ratchaburi province. The others are from Bangkok, Lopburi, Surin, Ubon Ratchathani, Sa Kaeo, Chanthaburi, Ranong, Chonburi, Nakhon Ratchasima, Kanchanaburi, Nan, Uttaradit and Chachoengsao province. Subgroup 2 contained seven samples, four samples from Trang province and three samples from Ranong. Subgroup 3 included five accessions from four provinces; Suphan Buri, Kanchanaburi, Sa Kaeo and Nakhon Ratchasima. Only one sample, accession no. 90 from Ubon Ratchathani, was positioned in subgroup 4. Accession no. 103 from Suphan Buri and no.106 from Kanchanaburi were situated in subgroup 5. Accession no.115, 116 and 117 from Trad province were clustered together in subgroup 6. Accession no. 120 from Sakon Nakhon was in subgroup 7. Accession no. 87 from Kanchanaburi is the only member of subgroup 8. Subgroup 9 contained one sample; accession no.88 from Chumphon. There are two samples in subgroup 10; accession no.37 from Prachuap Khiri Khan and no.38 from Kanchanaburi. Subgroup 11 has three accessions; no.66, 67 and 71 from Kanchanaburi. The only one sample, accession no.56 from Chiang Mai, was situated in subgroup 12.

Almost clustering of the *A. simplex* Bl. samples based on genetic similarity did not correlate with their region of origin, except for samples from Ratchaburi which were clustered together in subgroup 1 and all samples from Trad which were positioned in subgroup 6. This result suggested that geographical origin of the plant is not always a good factor to predict its genetic diversity. It is interesting to note that the samples from Kanchanaburi are widely distributed into six subgroups;1, 3, 5, 8, 10 and 11. This result may imply that *A. simplex* Bl population in Kanchanaburi has a high level of genetic variation.

4. Conclusions

The genetic diversity of Wan khanmak germplasm collected throughout Thailand was evaluated using AFLP marker. The AFLP profiles have proven to be efficient for genetic diversity evaluation of *Aglaonema* species. The results revealed that the germplasm collection of Wan Khanmak had great genetic diversity. The data obtained from this study are beneficial for further study on pharmacological and toxicological characterization, the management of genetic resources and conservation program.

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