Assessing genetic diversity of *Andrographis paniculata* (Burm. f.) Nees, an important medicinal plant of Thailand using RAPD markers

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ABSTRACT

"Fha-Tha-Laai-Joan" (*Andrographis paniculata* (Burm. f.) Nees is a medicinal plant widely used in Thailand and some other countries for therapeutic valued, but the lack of their genetic information may cause confusion in its utilization. The Randomly Amplified Polymorphic DNA (RAPD) markers were used to identify and elucidate the phylogenetic relationships among 58 accessions of *A. paniculata* collected throughout Thailand. Of thirty random primers tested, only eight primers generated a total of 66 bands, of which 26 bands were polymorphic, with an average of 10.5 bands per primer pair. Polymorphic information content (PIC) ranging from 0.034 to 0.405 with an average of 0.172 and 77.59% of *A. paniculata* accessions showed low PIC scores in a range between 0.00-0.068, indicating low diversity in these accessions. An effective number of allele per locus (n_e) as 1.018 and very low expected heterozygosity (H_e), with 0.21 were detected. Genetic similarities of 66 RAPD profiles were estimated via the Jaccard's coefficient and then the data were processed using UPGMA clustering method. The phylogenetic tree derived from RAPD data revealed that *A. paniculata* were divided into only one group in which the high similarity values were between 0.81-1.00. The results obtained can be indicated that the distributions of *A. paniculata* among each region are likely to belong to the same variety and are relatively undifferentiated across a large geographic range.

Keywords: *A. paniculata*, RAPD, genetic similarity

INTRODUCTION

Andrographis paniculata (Burm. f.) Nees, commonly known as Fha-Tha-Laai-

Joan, is a member of the Acanthaceae family (Figure 1) and has been widely used in health care traditions or Thai traditional medicine (TTM) in Thailand and other countries for the therapy in the common cold, fever and non-infectious diarrhoea (Caceres *et al*., 1999). In addition, past researches found various pharmacological activities of *A. paniculata*, including immune-stimulatory (Puri *et al*., 1993), anti-HIV (Otake *et al*., 1995; Calabrese *et al*., 2000), hepatoprotective (Handa and Sharm, 1990; Kapil *et al*., 1993), anti-malarial (Misra *et al*., 1992) and cardiovascular (Zhang and Tan, 1997 and Zhang *et al*., 1998)

Identification of *A. paniculata* cultivars in Thailand using many collecting samples from various locations through DNA-based techniques has been reported by using RAPD and SSCP (Maison *et al.*, 2005) and ISSR-Touchdown PCR (Sakuanrungsirikul *et al.*, 2008). The Randomly Amplified Polymorphic DNA (RAPD) markers are widely used for assessment of plant genetic relationship since they are a simple and rapid means used to detect polymorphic DNA sequences. The RAPD amplification technique is not much influenced by environment and thus surpassing the drawbacks generated by phenotypic plasticity (Williams *et al*., 1990).

This study used the arbitrary DNA amplification technique for assessment of genetic relationships among *A. paniculata* collected from different geographical locations of Thailand. This investigation was performed as a part of *A. paniculata* breeding program to find out their genetic variation. The backdrop of immense therapeutic value and lack of genetic information in Thai *A. paniculata* lead to sequential problem for their selection of divergent genotypes for crossing, effective conservation and management of its germplasm resources. This investigation will be insightful for further studies on germplasm for bioactive compound study and breeding program.

Figure 1 Plant sample of *A. paniculata* (Burm. f) Nees plant

MATERIALS AND METHODS

Plant materials

Fifty-eight accessions of *A. paniculata* were collected from five geograpic located throughout Thailand, consisting of eight accessions from the North, twenty-four from the Northeast, nine from Central, four from the East and eleven from the South (Table 1). All accessions were shown in the nursery of the greenhouse of Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkhen Campus.

Genomic DNA Extraction

Forty-five days after emergence, the three young leaves from each of the 58 *A. paniculata* accessions were sampled and subjected to DNA extraction. Total genomic DNA from the young leaves of *A. paniculata* was isolated using CTAB modified protocol (Murray and Thompson, 1980). Approximately 0.2 mg of young fresh leaves was ground in liquid nitrogen with a mortar and pestle. The ground tissue was placed in 1.5 ml microcentrifuge tube containing 500 µl of pre-heated extraction buffer (20 mM EDTA, 100 mM Tris-HCl, 1.4 M NaCl and 2% CTAB) and incubated at 65°C for 30 min. The samples were extracted with 500 µl of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube. The DNA was precipitated by adding a two-third of the volume of cold 90% ethanol, and then DNA was hooked and transferred to a new tube. The DNA was then washed with 70% ethanol and centrifuged at 10,000 rpm for 5 min. The ethanol was completely removed and the DNA pellet was dried by leaving the tubes uncovered at 37°C for 20-30 min. The DNA was dissolved in 150 µl TE buffer (10 mM Tris and 1.0 mM EDTA, pH 8). To confirm the concentration and quality of the DNA samples, DNA was run in 0.8 % agarose gel stained in 1 µg/ml of ethidium bromide solution and compared visually in Lambda DNA standards of known concentrations under ultra violet illumination. An aliquot of the isolated DNA for each sample was diluted to 20 ng/µl in TE buffer and stored at 4°C for use in subsequent assays, while the stock of DNA samples was stored at -40°C.

DNA analysis by RAPD marker

The RAPD amplifications were carried out using 30 random primers (Operon Technologies, Alameda, USA). RAPD reactions occurred in a total volume of 15 µl containing 200 µM of dNTPs, 2.5 mM $MgCl_2$, 0.3 U *Taq* DNA polymerase, 5 pmol of each primer and 50 ng of DNA. DNA amplification was performed in a DNA Thermal Cycler 600 (Applied Biosystems) in 0.2 ml PCR tubes programmed for initial denaturation at 95°C for 10 min, 44 cycles for 10 min at 95°C, 1 min at 35°C and 2 min at 72°C. The final extension step was carried out for 10 min at 72°C and the reactions were kept at 4°C. Amplification products were subsequently separated on 1.5% agarose gel electrophoresis.

Data analysis

To evaluate the efficiency of the RAPD markers in *A. paniculata* accessions identification, diversity and differentiation, we estimated the following for each assay unit (*U*) were calculated as reported by Morgante *et al.* (1994) used:

1) Number of assay units (*U*);

2) Average number of bands per assay unit (n/U);

3) Number of polymorphic bands (n_p);

4) Number of loci (L): in the case of RAPD markers, the theoretical maximum number of loci is equal to total number of bands ($n_p + n_{np}$);

5) Poly information content (PIC) or heterozygosities (H_n) = $1-\sum p_i^2$

6) Fraction of polymorphic loci (β) = n_p / n_p+n_{np}

7) Expected heterozygosity (H_e) = $\beta\sum H_{np}/n_p$

8) Effective number of alleles per locus (n_e) = $1/\sum p_i^2$, where p is the frequency of the ith allele;

9) Assay efficiency index (A_i) = N_e/U

Table 1 Geographical localities and abbreviations of the *A. paniculata* accessions studied.

Region	No. of accession	Origin of geographical distribution (Province)
Northern	8	Chiang Mai (APCM1, APCM2) Chaing Rai (APCR1, APCR2) Uttaradit (APUT) Lampang (APLP) Phayao (APPY) Sukhothai (APST1)
Northeastern	24	Nakorn Ratchasima (APNR1, APNR2) Surin (APSR) Buri Rum (APBR1, APBR2) Amnat Charoen (APAC) Ubon Ratchathani (APUB1, APUB2) Khon Kaen (APKK1, APKK2) Maha Sarakham (APMK) Si Sa Ket (APSK1, APSK2) Roi Et(APRE) Chaiyaphum (APCY1, APCY2) LOEI (APLO1, APLO2) Udon Thani (APUD1, APUD2) Nong Bua Lam Phu (APNB) Nong Khai (APNK1, APNK2) Kalasin (APKS)
Central	11	Kanchanaburi (APKN) Nakhon Pathom (APNP1, APNP2) Chachoengsao (APCC) Prachuap Khiri Khan (APPC1, APPC2) Phetchaburi (APPB) Prachin Buri (APPR1, APPR2) Sing Buri (APSB) Saraburi (APSA)
Eastern	4	Trat (APTD) Chanthaburi (APCT) Rayong (APRA1, APRA2)
Southern	11	Chumphon (APCP) Satun (APST) Songkhla (APSO1, APSO2) Phatthalung (APPL) Trang (APTR) Krabi (APKB1, APKB2) Phangnga (APPN) Nakhon Si Thammarat (APNS) Surat Thani (APSRT)
Total	58	

The RAPD profiles generated were score as discrete variables using 1 to indicate presence and 0 to 1 to indicate the absence of a band. Binary data accessions were evaluated by estimating the Jaccard's coefficient (Jaccard, 1908), and the accessions were clustered by the unweighted pair-group method with arithmetic averages (UPGMA). The dendrograms generated on the basis of the above indexes were compared by computing the co-phenetic correlation (Sneath and Sokal, 1973). Genetic similarity among all accessions was calculated using the NTSYS-pc program.

RESULTS AND DISCUSSION

Thirty primers were used for preliminary screening. Only eight primers (~26%) produced a few polymorphic bands and thus used to evaluate the remaining varieties. A summary of all primers and amplified products from this study is shown in Table 1.

Level of polymorphism and power of discrimination

Eight primers were scored for their consistent reproducible amplification and reproducible band criteria in three replicated PCRs across 58 *A. paniculata* accessions. A total of 66 bands were amplified using the 8 oligonucleotide selected primers. All of the 8 decamer RAPD primers could amplify DNA from all *A. paniculata* accessions and allowed the intraspecific differentiation of their accessions, with a total of 26 polymorphic bands. On average 8.25 DNA fragments were found per primer and the mean number of bands per *A. paniculata* accession was very low. The 37.63 percentages of polymorphic bands were given from all of RAPD primer (Table 2). The RAPD profiles generated by the primer OPZ-04 are shown in Figure 2. The amplified products were then categorized based on their size ranging from 500 to 1,900 bp and the number of bands produced ranged from 3 using OPZ-08 to 12 using OPW-05 primer (Table 2). Out of 66 bands observed 31 (56.6%) were monomorphic for all accessions examined in this study. Percentages of polymorphic bands for each primers and polymorphic information content (PIC) for each marker were shown in Table 2. Primer OPW-05 exhibited the greatest level of polymorphism (41.66%). Analysis of 66 RAPD loci among all cultivars of *A. paniculata* accessions showed that the PIC among all polymorphic loci showed a little polymorphic DNA ranged from 0.034 to 0.405.

An effective number of allele per locus (n_e) as 1.018, could be detected for RAPD. This value was reflected also in very low heterozygosity (H_e), with 0.21. Furthermore, the very low assay efficiency index (A_i) values also were observed for RAPD in this study, with 8.25 as shown in Table 3. The co-phenetic correlation coefficients provided for RAPD marker in our study indicate the extent to which the clustering of genotypes depicted in the trees accurately represents the estimates

of genetic similarity between *A. paniculata* accessions obtained with that marker system. The co-phenetic of RAPD analysis reflected at 0.938 (r = 0.938).

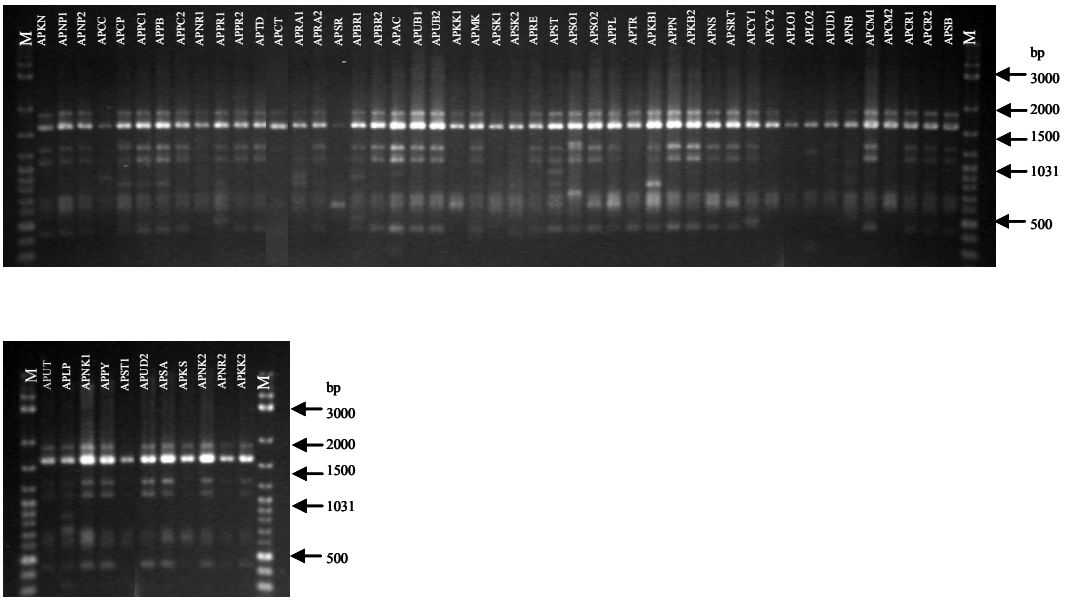

Figure 2 Monomorphic pattern of RAPD profile from the genomic DNA of *A. paniculata* generated by primer OPW-05. Lane M, molecular marker (GeneRuler ^{TM} DNA Ladder Mix).

Table 2 RAPD primers used for *A. paniculata* identification by RAPD, the number of bands produced and polymorphic band percentages

Primer	5′-sequence-3′	GC (%)	No. of amplified bands	No. of polymorphic bands	polymorphic bands (%)	PIC
OPZ-01	TGTGTGCCAC	60	11	4	36.36	0.1683
OPZ-04	AGGCTGTGCT	60	10	1	10	0.0339
OPZ-06	GTGCCGTTCA	60	7	2	28.57	0.0678
OPZ-08	GGGTGGGTAA	60	3	1	33.33	0.0666
OPZ-10	CCGACAAACC	60	11	7	63.63	0.4047
OPZ-12	TCAAGGGGAC	60	4	1	25	0.0339
OPZ-16	TCCCCATCAC	60	8	5	62.50	0.2349
OPW-05	CTGCTTCGAG	60	12	5	41.66	0.3621
	Total bands		66	26		
	No. of bands per primer		8.25	3.25		

Phylogenetic relationship among *A. paniculata* accessions

Pairwise comparisons among all accessions were made and genetic similarity values were estimated base on eight decamer RAPD primers. The mean of genetic similarity for all accessions were high at 0.905 with variation ranging from 0.809 to 1.000.

The data obtained from a genetic similarity values were then used to determine the genetic relationship of *A. paniculata.* The UPGMA cluster analysis based on the

Jaccard's coefficient was performed and used to construct the phylogenetic tree. All of the accessions of *A. paniculata* collected from the five geographic locations in Thailand were grouped as one cluster at an arbitrary cut-off of 87% similarity level on the phylogenetic tree, which is shown as a dendrogram in Figure 3. These results indicated that all of the samples collected from various geographic locations of Thailand were likely to be of the same variety.

Table 3 Level of polymorphism obtained with RAPD markers in 58 samples of *A. paniculata*

Parameters	
Number of assay units	8
Average number of bands per assay unit	8.25
Number of polymorphic bands	26
Number of bands	66
Average Polymorphic information content (PIC)	0.172
Expected Heterozygosity (*He*)	0.21
Effective number of alleles per locus (n_e)	1.018
Assay efficiency index (*Ai*)	0.145

In this experiment, the distribution of *A. paniculata* among the regions was relatively undifferentiated across a broad geographic range. It is interesting to note from previous reports that *A. paniculata* is probably native to India and has been introduced and cultivated as a medicinal plant in many parts of Asia (Widen *et al*., 1992). This result further supported the hypothesis that *A. paniculata* is an inbreeder species.

The result of the current study revealed very low level of genetic variability detected across all accessions using RAPD markers. However, this study corresponded to those of Padmesh *et al*. (1998) which reported that *A. paniculata* accessions from Thailand were closely related to those from India. *A. paniculata* of Indian and Thai provenances possibly have a common origin. Having a genotype different from other accessions suggests that *A. paniculata* from Thailand is geographically distant from the rest. This result also corresponded with Maison *et al*. (2005) who reported that the genetic similarity among 25 Thai *A. paniculata* accessions using RAPD was more than 67%, and the average similarity coefficient for the population was 0.83, indicating a high genetic relatedness. However, even though the results in the current revealed the similar pattern of genetic variation distribution in this species located throughout Thailand. This study has preferable results with higher amounts of the number of accessions and more comprehensive covering across the country. In addition, Maison *et al.* (2005) also reported that SSCP analysis showed little polymorphism of specific amplified products existing among these

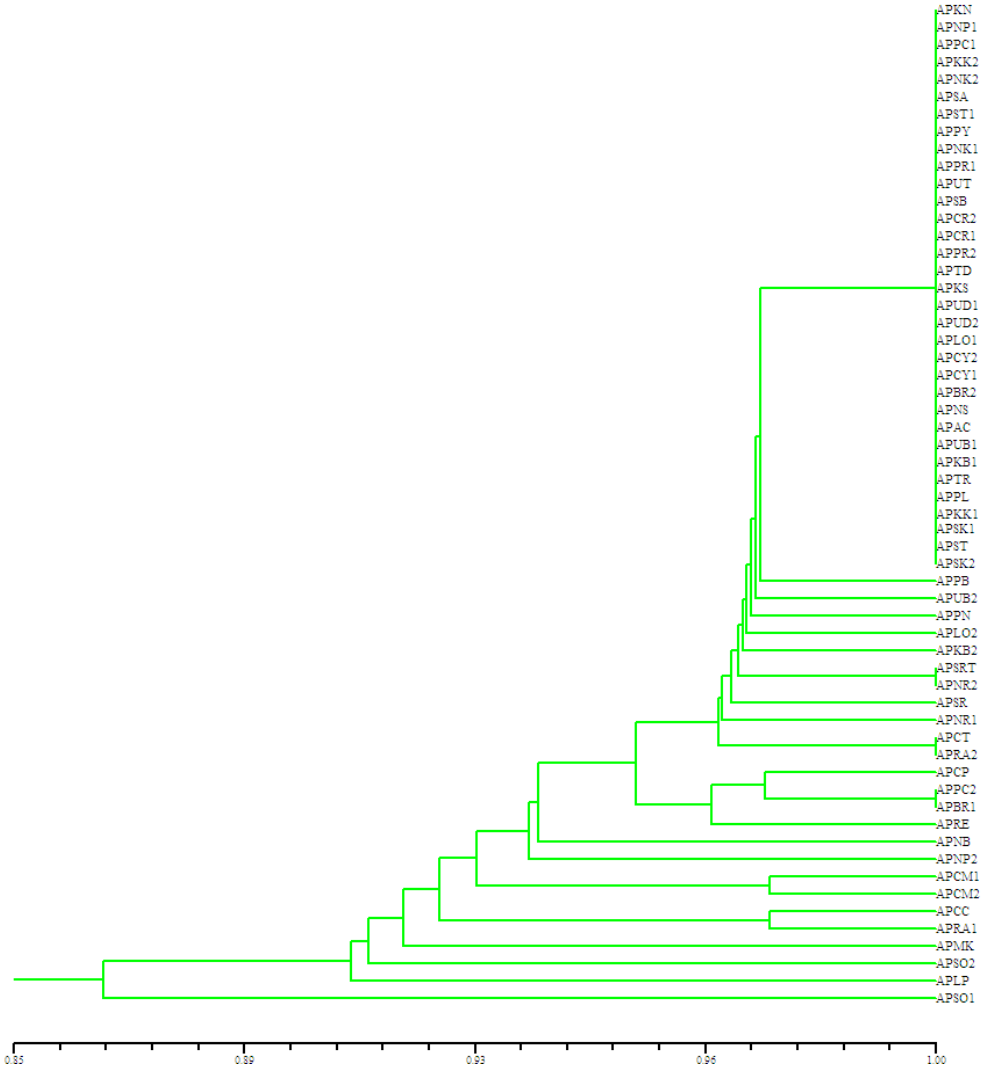

Figure 3 Dendrogram illustrating genetic relationship among 58 *A. paniculata* accessions generated by UPGMA cluster calculated from 66 bands of RAPD markers amplified by 8 Operon primers as listed in Table 2. The genotypes are listed and defined as in Table 1.

accessions. Furthermore, similar results using molecular markers were reported by Sakuanrungsirikul *et al.* (2008) showing no genetic variation among *A. paniculata* accessions from nine areas throughout Thailand. It was reported that all loci generated by ISSR-Touchdown PCR and high annealing temperature RAPD techniques were found to be monomorphic across all accessions. The results in the current study also corresponded with Lattoo *et al*. (2006) and Kumar and Shekhawat (2009) who reported high similarity values based on RAPD markers from 53 *A. paniculata* accessions in five ecogeograpic regions of India. They suggested that the extent of genetic diversity observed in each region was in conformity with the breeding behavior of the species. As previously reported, in all studies involving genetic variability analysis,

including the current study, all DNA-based molecular markers techniques (RAPD, SSCP, ISSR, markers) have produced similar results with *A. paniculata* and no genetic variation has been detected. It has been well explained that *A. paniculata* accessions collected from different parts of the country, limited genetic diversity exists among these accessions. Thus, it is possible that there is no genetic variation due to the species being hermaphroditic, self-compatible and a habitual inbreeder. Intimate proximity of ad pressed stigma to the anthers, and synchronization of anther dehiscence and stigma receptivity, provide for obligate autonomous, self pollination in the species, as found in a previous report by Lattoo *et al*. (2006).

In conclusion, all of 58 collecting samples of *A. paniculata* in this study revealed extremely high genetic similarity. Although some amount of variability was detected with RAPD markers, it did not warrant separation into several clusters. The technique employed in this study has the advantage of being inexpensive to perform, and does not require a previous knowledge of the genome. The similar results of previously reported for genetic variability analysis including the current study, RAPD markers could be appropriate for analysis of genetic variability in closely related genotypes.

Further research is needed on the use of mutations to induce genetic variability of *A. paniculata* in the Thai germplasm. Analysis of the sequencing of any mutated genes could be used in the breeding program for optimizing its genetic amelioration and also to enrich bioactive compounds.

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