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Genetic Diversity of *Andrographis paniculata* Wall. ex Nees as Revealed by Morphological Characters and Molecular Markers

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ABSTRACT

Intraspecific variation of twenty-five accessions of *Andrographis paniculata* Wall. ex Nees, collected from different geographical areas in Thailand and one from Laos, was determined by morphological characters and molecular analysis using RAPD and SSCP techniques. Morphological characters and total lactone content variation were measured at 50% flowering time among the accessions grown under uniform environmental condition. UPGMA cluster analysis of all accessions resulted in 4 major groups based on 18 morphological characters while RAPD analysis distinguished 5 groups at 0.82 of similarity coefficient, using 247 bands generated from 14 primer amplifications separated on polyacrylamide gel. Morphological characters and RAPD were incongruent and were not correlated to geographical area of collection and yield of active compound. SSCP analysis showed little polymorphism of specific amplified products. Markers for the *CPS1-2*, *IDH1*, *IDH2* and *IPI* genes were monomorphic and only 2 alleles were detected for *CAT* and *CPS1-1* genes.

Key words: *Andrographis paniculata*, diversity, molecular marker

INTRODUCTION

Andrographis paniculata is an erect annual herb with extremely bitter taste in all parts of the plant. It is known in Thailand as "Fathalaichon", and is recommended by the National Primary Health Care Programme to treat fever, sore throat, dysentery and diarrhoea (Department of Medical Sciences, 1999). Nowadays, it is also used in the form of "Andrographis gel" for treating periodontitis. Furthermore, the plants are also used as feed supplement in large scale industrial chicken and pig farms.

A major problem for the use of this herb in medical treatments is the lack of standard amounts of andrographolides in crude drug from different geographical sources which affect on the quality of the pharmaceutical production and its therapeutic efficacy. Consequently, the present study is performed to determine genetic diversity of *Andrographis paniculata* collected from different locations using morphological characters, chemical constituents and molecular markers and to generate a database on *Andrographis* which can be used for crop improvement program.

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Considerable variation in morphological characters, biochemical characters and isozyme patterns have been reported previously in *Andrographis paniculata* (Sabu *et al.*, 2001). However, morphological and isozyme marker could be influenced by the stages of plant growth as well as environmental factors resulting in inconclusive interpretation. Only morphological or biochemical alone do not provide sufficient information to fully understand the genetic diversity within the species. Nowadays, a large number of DNA-based molecular markers techniques have been developed such as RFLPs (restriction fragments length polymorphism) (Botstein *et al.*, 1980), AFLPs (amplified frangments length polymorphism) (Vos *et al*., 1995), RAPDs (random amplified polymorphic DNA) (Williams *et al.*, 1990; Kazan *et al*.,1993) and SSCP (single-strand conformation polymorphism) (Orita *et al.*, 1989). Among these marker techniques, RAPD-based molecular markers have been found to be useful in *Andrographis paniculata* (Padmesh *et al.*, 1998). It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles. Many researchers have studied geographical variation at genetic level. Estimates of genetic diversity are also important in designing crop improvement programmes for management of germplasm and developing conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana* (Shasany *et al*., 1999), *Allium schoenoprasum* L. (Friesen *et al.*, 1999 and Blattner, 2000) collected from differrent geographical regions. Some studies on vetivers (Pomthong, 2002), and *Cuphea* (Slabaugh *et al.*, 1997) suggest that SSCP might be appropriate for analysis of genetic variability in closely related genotypes. The objective of the present study was to evaluate genetic diversity of *Andrographis paniculata* using morphological characters, concentration of biochemical constituents, RAPD and SSCP techniques.

MATERIALS AND METHODS

1. Plant materials

Twenty two accessions of seeds (No. 1-22) collected from different parts of Thailand were grown in the same field plot at Kamphaeng Saen campus for morphological investigation. Four accessions (No. 23-26) were added later for molecular study. Sources of seeds are shown in Table1.

Table 1 Accessions of *Andrographis paniculata* from different locations.

No.	Accession	No.	Accession
1	Kamphaeng Saen, Nakhon Pathom	14	Phetchaburi
2	Kamphaeng Saen, Nakhon Pathom	15	Buri Ram
3	Kamphaeng Saen, Nakhon Pathom	16	Songkhla
4	Kamphaeng Saen, Nakhon Pathom	17	Rayong1
5	Kamphaeng Saen, Nakhon Pathom	18	Phitsanulok
6	Kamphaeng Saen, Nakhon Pathom	19	Rayong2
7	Kamphaeng Saen, Nakhon Pathom	20	Si Sa Ket
8	Kanchanaburi	21	Pak Chong, Nakhon Ratchasima1
9	Chanthaburi	22	Pak Chong, Nakhon Ratchasima2
10	Chiang Rai	23	Chachoengsao1
11	Chiang Mai	24	Chachoengsao2
12	Chumphon	25	Laos PDR
13	Nakhon Si Thammarat	26	Uttaradit

2. Morphological analysis

Morphological variation was measured on 20 plants of each accession grown under the same conditions. Sixteen phenotypic characters were measured at 50% flowering time: plant height, bush width, number of node, internode length, branch length, leaf length, leaf width, flower length, flower width, fresh leaf mass, dry leaf mass, fresh stem mass, dry stem mass, day to first flower, harvesting ages and total lactone content. A distance matrix was calculated using EUCLIDSQ (Euclidean distances squared) coefficient and UPGMA clustering.

3. Phytochemical analysis

Five plants from the same accession were harvested on the same day, when the average percentage of blooming flowers within each accession was about 50%. The total lactone content was measured using the modified method from Jewvachdamrongkul *et al*. (1990).

4. Molecular analysis

Total genomic DNA from the young leaves of one plant per accession was extracted following the procedure outlined in the Qiagen DNeasy® Plant Mini Kit Handbook.

4.1 RAPD technique

Total 20 random decamer primers (Operon Technologies, USA) were used to amplify frangments from four *Andrographis* DNA. The PCR reaction of 25 µl contained 20 ng of template DNA, 1 mM of each dNTP, 1x PCR buffer, 2mM $MgCl_2$ and 0.5U of Taq DNA polymerase (Fermentas, Lithuenia) and 0.3 µM random decamer primer. PCR product was conducted in a thermal cycle programmed as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 37°C for 45 s and extension at 72°C for 1 min. After 35 cycles, the program was allowed a final extention at 72°C for 5 min before maintaining at 4 °C. Amplified products were separated on 1.5% agarose gel in TAE buffer. Electrophoresis was performed at 200 V for 20 min. The gel was stained in 1 µg/ml ethidium bromide solution for 10 min, visualized with ultraviolet transillumination, and photographed with a gel documentation system. The size of amplified products was determined by comparison with 100 bp DNA Ladder. Those primers that gave many and clear amplified bands were used further to amplify among all accessions. Amplified product of 26 accessions were separated on 10% polyacrylamide gel in TBE buffer at 200 V for 4 h and stained by silver nitrate for polymorphism determination. Only clearly distinguishable RAPD amplified bands were included for analysis. Genetic similarity among all accessions was calculated using the simple matching (SM) coefficients (Legendre and Legendre, 1983) and UPGMA clustering within NTSYS-pc version 2.01.

4.2 Specific nuclear markers

Markers were developed using degenerated PCR primers that anneal to conserved regions in specific plant genes related with terpene pathway: gene coding for isopentenyl diphosphate isomerase (IPI), copalyl diphosphate synthase (CPS), geranylgeranyl diphosphate synthase (GGPP synthase). In addition, fragments from some general metabolic genes (IDH, CAT) were isolated. *Andrographis* DNA from twenty-six accessions were amplified using specific primer, each reaction contained in 15 µl volume: 20 ng of template DNA, 4 pmol of each primer, 1 mM of each dNTP, 1x PCR buffer, 2mM $MgCl_2$ and 0.5U of Taq DNA polymerase (Fermentas, Lithuenia). Cycling condition was start 94°C for 3 min; then 35 cycles of 94°C for 45 sec, 50°C for 45 sec, 72° C for 1 min and final extention of 72°C 5 min. The quality of the amplification was checked by electrophoresis on 0.8% agarose gel in TAE buffer at 200 V for 20 min and then stained in 1 µg/ml ethidium bromide solution for 10 min, visualized under ultraviolet transillumination, and

photographed, comparing with a precision molecular mass *Hin*dIII/*Eco*RI DNA marker (Fermentas, Lithuenia). The amplified products from six specific primers (Table 6) were separated on 6% non-denaturing polyacrylamide gel in TBE buffer at 200 V for 18 h. The gel was stained with 1.5% silver nitrate. The obtained fragments of more than 700 bp in size (CPS1 and IDH2) were digested with a restriction enzyme (*Hin*dIII and *Xba*I) and determined by electrophoresis on 6% non-denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Morphological characters analysis

Cluster analysis of morphological characters based on Euclidean distance matrix was used to generate a dendrogram (Fig.1) with 4 clusters at the distance coefficient values of 2830. Cluster A included Kamphaeng Saen(1), Nakhon Si Thammarat, Phetchaburi and Rayong2. Cluster B contained a large group of 12 accessions, which were Kamphaeng Saen(2-7), Chumphon, Si Sa Ket, Phitsanulok, Chantaburi, Chiang Rai and Kanchanaburi. Cluster C consisted of five acessions that were Chiang Mai, Buri Rum, Songkhla, Rayong1 and Pakchong1. Cluster D included only Pakchong 2. The average distance coefficient for Kamphaeng Saen population was 2012.6 while the rest was 3215 and for all population was 3145.9 (Table 3). The grouping of 22 accessions of *Andrographis* based on 17 morphological characters and total lactone content were not correlated with the accession collected site and total lactone content. Sabu *et al*. (2001) reported the similar result based on morphological characters

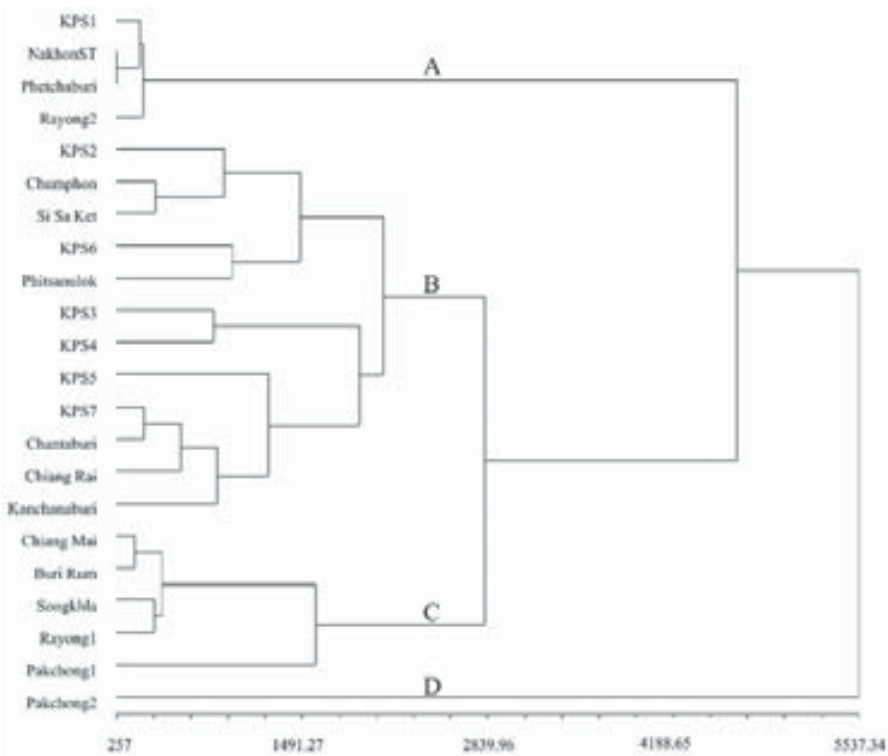

Figure 1 Dendrogram depicting the phenetic relationship of 22 accessions of *Andrographis paniculata* based on morphological characters, using distance coefficient by Euclidean distances squared, clustering with UPGMA.

with accessions from different geographical origins clustering in the same group. A possible explanation for this was that the seeds were collected from the same geographical origin and were transferred for cultivation in many regions of Thailand.

Phytochemical analysis

Total lactone content varied within the range from 1.1-13.8% (Table 2) among 22 accessions collected from different location in Thailand. Although bringing them together and grown under the same condition, the active compound still had high variation. The result indicated that genetic differences might affect the production of this phytochemical compound in Fathalaichon.

RAPD analysis

Twenty random decamer primers were tested to produce DNA polymorphism in genomic DNA of *Andrographis paniculata*. Six primers generated amplification product with a low number of bands on agarose and were not used further. The remaining fourteen primers (Table 4) were analysed on polyacrylamide gel, produced 247 amplification fragments (500 –1400 bp in size) from all *Andrographis* accession with an average of 17.6 bands per primer, 141 bands were polymorphic with an average of 12.2 bands per primer. The primer OPA-16 appeared more polymorphism (Fig. 3). The similarity coefficients of RAPD data of 26 accessions ranged between 0.67-0.95 (Table 5). At 0.79 similarity level on the dendrogram, the accessions were separated into 5 groups (Fig. 2). Cluster A included KPS1 which was separated from the other KPS accessions. Cluster B contained most of the accessions including KPS2-KPS7, Buri Ram, Kanchanaburi, Chanthaburi, Chiang Rai, Chiang Mai, Nakhon Si Thammarat, Rayong 1, Phitsanulok, Rayong 2, Si Sa Ket, Phetchaburi, Songkhla, Chachoengsao 1 and Chachoengsao 2. Accessions from Pakchong1 and Pakchong 2 constituted cluster C while accessions from Laos and Uttaradit were separated in cluster D and E, respectively. The average similarity coefficient for Kamphaeng Saen population was 0.86 while the rest was 0.83 and for all population was 0.83. The similarity among Thai accessions was more than 67% suggesting that although they were collected from different parts of the country, limited genetic diversity exists among these accessions. The genetic similarity of Kamphaeng Saen seed and the rest of the populations might be due to seed transferred by local grower in various provinces. This result corresponded to the report of Singh *et al*. (2001) that *Podophyllum hexandrum* from different regions of Kullu and Chamba indicated high genetic relatedness. The similar study of *Andrographis* (Sabu *et al.*, 2001) revealed an exotic and Tamil Nadu accessions clustered together with Kerala accessions.

SSCP technique

The amplified products of 26 accessions using six specific primers showed that markers for the *CPS1-2, IDH1, IDH2, IPI* genes were monomorphic. Only 2 alleles were detected for *CAT* and *CPS1-1* gene showing the polymorphism among *Andrographis* accessions (Fig. 4). The results indicated low genetic diversity in *Andrographis paniculata* collected from different locations in Thailand. Padmesh *et al*. (1998) reported that the accessions from Thailand were closely related to the samples from India. *Andrographis paniculata* of Indian and Thai provenances possibly have a common origin. SSCP is capable of detecting single nucleotide mutation but SSCP markers using specific primer in present study may not be suitable for estimating genetic similarity between very closely related accessions of *Andrographis paniculata*.

The DNA sequences obtained from the clones of *Andrographis* specific genes were submitted to GenBank: *CAT* (AJ973128), *CPS* (AJ973129), *IDH* locus1 (AJ973130), *IDH* locus2 (AJ973131), *IPI* (AJ973132), *GGPS* locus1 (AJ973133), *GGPS* locus2 (AJ973134).

Table 2 Eighteen morphological characters among 22 acessions of *Andrographis* paniculata, grown under the same condition.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
stem height (cm)	45.67	53.10	51.75	49.53	43.46	51.77	46.88	47.18	53.06	55.54	51.37	53.32	46.63	47.33	57.03	57.12	59.18	58.92	42.83	59.03	49.85	62.20
bush width (cm)	27.67	41.50	42.40	38.16	29.64	42.73	37.90	36.87	43.06	41.17	44.93	35.71	32.25	29.67	47.83	46.69	47.94	46.33	25.56	43.27	24.46	42.27
node number/plant	16.00	15.85	15.50	14.11	14.64	15.36	15.55	14.74	15.94	16.83	16.13	15.86	14.75	15.33	15.92	16.50	16.82	17.33	12.89	17.40	15.08	16.67
10th internode length(cm)	4.00	4.00	4.13	3.99	3.77	4.18	3.52	6.00	4.16	3.95	3.95	4.04	4.38	3.60	4.30	4.38	3.95	4.38	4.59	4.61	3.97	4.40
10th branch length(cm)	9.50	16.80	16.28	14.11	15.61	17.09	17.68	15.05	19.13	23.23	28.00	21.26	18.75	10.67	24.83	21.67	23.28	25.00	12.22	21.47	15.31	23.14
10th leaf width(cm)	0.63	0.96	1.05	0.92	1.14	0.97	1.22	1.16	1.18	1.31	1.76	1.05	0.68	1.27	1.38	1.29	1.51	1.27	0.78	1.06	1.21	1.43
10 th leaf length(cm)	3.40	4.97	5.20	4.53	5.35	5.00	5.62	4.54	5.78	6.22	7.25	5.43	4.00	6.33	6.43	6.23	5.94	6.67	4.34	5.73	5.73	8.47
flower width (cm)	0.53	0.51	0.49	0.48	0.54	0.49	0.53	0.51	0.53	0.51	0.61	0.55	0.53	0.50	0.46	0.50	0.49	0.57	0.53	0.55	0.55	0.54
flower length (cm)	1.50	1.51	1.48	1.52	1.47	1.51	1.50	1.40	1.52	1.43	1.44	1.48	1.50	1.50	1.43	2.24	1.48	1.48	1.47	1.51	1.49	1.51
leaf fresh mass (g)	6.33	16.27	13.20	12.53	14.23	20.08	15.57	28.60	19.42	21.74	35.28	25.14	11.67	12.00	29.30	27.62	27.58	26.17	9.50	18.30	7.62	29.80
leaf dry mass (g)	1.63	2.75	8.83	5.81	4.27	10.67	3.69	2.46	5.55	7.09	9.08	8.55	2.39	2.52	6.47	7.32	8.40	7.64	2.86	6.17	2.02	5.87
%leaf dry mass	25.75	16.90	66.89	46.37	30.01	53.14	23.70	8.60	28.58	32.61	25.74	34.01	20.48	21.00	22.08	26.50	30.46	29.19	30.11	33.72	26.51	19.70
stem fresh mass (g)	16.33	49.45	36.30	29.53	31.00	61.70	32.64	46.33	44.25	51.94	66.25	48.00	22.33	27.33	78.08	67.54	84.67	67.67	15.25	61.20	14.38	63.20
stem dry mass(g)	4.88	13.07	15.97	12.74	10.95	26.39	10.37	11.18	14.08	18.90	21.84	17.46	7.38	7.35	21.92	29.39	32.78	22.06	4.55	24.77	4.31	24.46
%stem dry mass (g)	29.88	26.43	43.99	43.14	35.32	42.77	31.77	24.13	31.82	36.39	32.97	36.38	33.05	26.89	28.07	43.51	38.72	32.60	29.84	40.47	29.97	38.70
Day to 1 flower	214	198	198	184	184	184	184	184	184	196	184	210	221	221	184	184	184	184	228	214	162	162
harvest age(day)	229	229	215	204	225	229	204	204	197	204	197	225	236	236	198	204	198	229	236	229	177	173
% total lactone	11.85	10.41	12.28	8.56	8.26	9.62	13.81	13.27	8.01	9.59	9.64	11.90	11.32	10.97	9.97	7.99	13.28	1.11	8.73	13.28	10.65	13.54

Table 3 Distance matrix of 22 accessions of *Andrographis* from morpholoigical characters by EUCLIDSQ coefficient.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	0.0																					
2	1914.7	0.0																				
3	3268.5	3238.0	0.0																			
4	2579.8	2426.6	848.7	0.0																		
5	1350.1	1055.5	2056.0	893.3	0.0																	
6	4937.8	2185.0	1395.1	2010.5	2107.9	0.0																
7	2101.1	1251.8	2457.8	723.2	614.5	2848.9	0.0															
8	3415.4	1133.8	4569.3	2376.7	1566.5	3640.3	662.0	0.0														
9	3391.6	1445.6	2281.6	826.7	1299.0	2239.3	340.8	737.3	0.0													
10	3252.7	1114.6	1761.5	1145.3	1503.3	1456.1	876.1	1210.6	350.4	0.0												
11	6300.4	2248.1	3910.6	2793.2	3093.2	2276.3	1930.3	1296.8	907.9	708.7	0.0											
12	1971.1	741.1	1737.6	1932.0	1342.7	1471.1	1709.1	2089.2	1651.4	715.8	2138.7	0.0										
13	317.0	1558.2	3695.3	3331.5	1722.4	4773.1	2580.8	3533.0	3726.7	3132.2	5943.4	1482.9	0.0									
14	308.1	1348.8	3781.1	3445.2	1724.9	4614.0	2601.2	3291.3	3661.0	3090.5	5812.2	1426.3	142.6	0.0								
15	7316.1	2390.7	4930.0	3849.8	4019.3	2648.7	2715.7	1704.8	1437.0	1160.3	272.4	2740.8	6884.7	6544.6	0.0							
16	6085.5	2023.8	3412.4	2544.0	2903.5	1517.6	2118.4	1778.5	1074.5	659.1	362.5	1908.4	5693.0	5587.1	480.7	0.0						
17	8700.5	3411.3	4808.2	4288.8	4964.3	2258.8	3791.4	3044.1	2217.5	1562.6	682.9	3199.8	8202.3	7895.2	370.6	418.2	0.0					
18	5172.3	1136.1	3407.2	3106.3	2312.3	983.3	2583.5	2215.3	1817.0	1186.1	1266.1	1417.8	4509.9	4307.6	1238.0	870.6	1561.2	0.0				
19	317.4	2815.9	3917.8	3899.6	2434.1	5896.3	3612.9	5010.9	4971.6	4363.9	7807.4	2334.4	327.2	356.4	8974.0	7617.6	10362.8	6285.8	0.0			
20	3323.5	1098.4	2381.5	3078.8	2575.7	1399.2	2933.5	3181.5	2499.8	1134.2	2497.1	423.5	2496.5	2397.3	2614.2	1781.1	2606.9	1266.7	3684.5	0.0		
21	5484.1	5791.3	5591.1	2316.0	3271.8	7360.1	1865.8	3249.9	2409.7	4190.9	5293.4	6534.4	7183.9	7253.9	6496.0	5877.0	7919.3	7484.3	7926.0	8801.0	0.0	
22	9805.7	5248.7	6565.6	4051.6	5379.7	5053.2	3142.6	2596.6	1899.7	2579.8	1351.2	5665.5	10176.7	10047.6	1534.1	1648.4	1814.3	3978.4	12457.3	6198.2	3984.5	0.0

X0 = overall average coefficient of distance = 3145.9

X1 = average coefficient of distance among Kamphaeng Saen populations = 2012.6

X2 = average coefficient of distance among the rest of the populations = 3215

Table 4 Fourteen decamer RAPD primers (Operon Technologies, USA), sequence and products generated through amplification.

Primer	Sequence (5'-3')	Number of amplified bands	Number of polymorphic bands
OPA-01	CAGGCCCTTC	17	7
OPA-03	AGTCAGCCAC	14	5
OPA-04	AATCGGGCTG	15	5
OPA-05	AGGGGTCTTG	21	15
OPA-07	GAAACGGGTG	17	9
OPA-09	GGGTAACGCC	24	14
OPA-10	GTGATCGCAG	24	16
OPA-13	CAGCACCCAC	19	10
OPA-14	TCTGTGCTGG	20	16
OPA-15	TTCCGAACCC	13	8
OPA-16	AGCCAGCGAA	20	12
OPA-18	AGGTGACCGT	13	3
OPA-19	CAAACGTCGG	12	8
OPA-20	GTTGCGATCC	18	13
Total		247	141

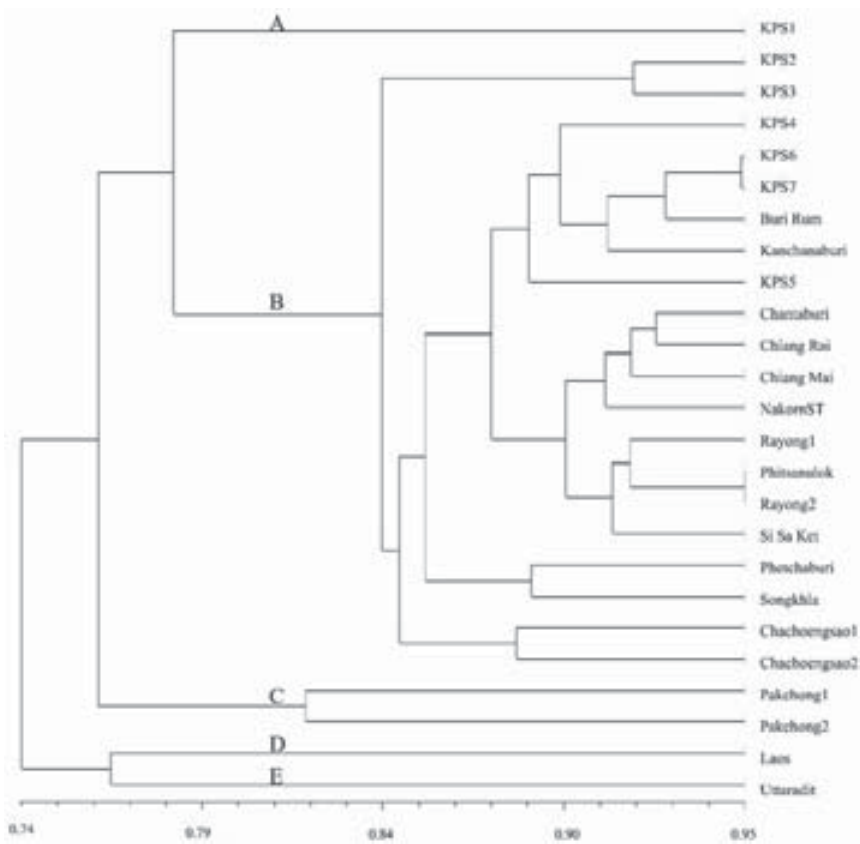

Figure 2 Dendrogram depicting the phenetic relationship of 25 accessions of *Andrographis paniculata* based on RAPD technique, using similarity coefficient by simple matching, clustering with UPGMA.

Table 5 Similarity matrix of 26 accessions *Andrographis* based on RAPD by simple matching coefficient.

No.	1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	1.00																								
2	0.85	1.00																							
3	0.82	0.92	1.00																						
4	0.77	0.89	0.90	1.00																					
5	0.81	0.85	0.84	0.88	1.00																				
6	0.79	0.88	0.88	0.90	0.90	1.00																			
7	0.82	0.84	0.87	0.89	0.89	0.95	1.00																		
8	0.77	0.86	0.86	0.91	0.89	0.91	0.92	1.00																	
9	0.79	0.81	0.82	0.83	0.80	0.87	0.88	0.85	1.00																
10	0.77	0.83	0.85	0.84	0.85	0.90	0.91	0.88	0.93	1.00															
11	0.75	0.83	0.88	0.87	0.83	0.88	0.90	0.88	0.92	0.92	1.00														
13	0.76	0.81	0.85	0.86	0.86	0.91	0.93	0.88	0.90	0.92	0.92	1.00													
14	0.76	0.81	0.80	0.81	0.82	0.86	0.88	0.85	0.88	0.88	0.86	0.88	1.00												
15	0.77	0.84	0.86	0.89	0.88	0.92	0.94	0.91	0.86	0.89	0.87	0.89	0.84	1.00											
16	0.78	0.81	0.83	0.83	0.84	0.88	0.87	0.88	0.84	0.85	0.85	0.86	0.89	0.86	1.00										
17	0.76	0.83	0.85	0.85	0.87	0.87	0.87	0.89	0.88	0.88	0.89	0.87	0.85	0.88	0.88	1.00									
18	0.78	0.85	0.86	0.87	0.86	0.89	0.89	0.89	0.90	0.90	0.91	0.90	0.88	0.88	0.87	0.92	1.00								
19	0.78	0.85	0.88	0.88	0.86	0.90	0.91	0.88	0.92	0.91	0.93	0.92	0.86	0.90	0.87	0.92	0.95	1.00							
20	0.79	0.85	0.88	0.88	0.87	0.90	0.91	0.89	0.88	0.91	0.92	0.88	0.84	0.89	0.86	0.90	0.90	0.93	1.00						
21	0.76	0.76	0.78	0.77	0.77	0.79	0.81	0.80	0.78	0.83	0.82	0.81	0.77	0.78	0.76	0.81	0.82	0.85	0.85	1.00					
22	0.69	0.67	0.67	0.72	0.71	0.73	0.72	0.74	0.69	0.73	0.73	0.75	0.77	0.71	0.78	0.73	0.72	0.73	0.75	0.82	1.00				
23	0.77	0.82	0.82	0.87	0.85	0.85	0.87	0.86	0.84	0.88	0.87	0.88	0.82	0.89	0.82	0.88	0.88	0.88	0.90	0.81	0.75	1.00			
24	0.77	0.81	0.82	0.87	0.84	0.85	0.83	0.83	0.80	0.83	0.83	0.84	0.77	0.84	0.79	0.83	0.86	0.88	0.86	0.80	0.75	0.88	1.00		
25	0.69	0.71	0.73	0.76	0.74	0.77	0.77	0.74	0.76	0.79	0.76	0.77	0.72	0.78	0.72	0.77	0.79	0.80	0.79	0.79	0.72	0.78	0.83	1.00	
26	0.70	0.71	0.69	0.72	0.67	0.72	0.71	0.72	0.73	0.69	0.73	0.71	0.71	0.67	0.71	0.72	0.73	0.75	0.72	0.74	0.75	0.73	0.80	0.77	1.00

X_0 = overall average coefficient of similarity = 0.83

X_1 = average coefficient of similarity among Kamphaeng Saen populations = 0.86

X_2 = average coefficient of similarity among the rest of the populations = 0.83

Table 6 PCR products amplified from six *Andrographis* specific primers.

Locus	Specific primer	Sequence 5'-3'	Digestion	Number of alleles
CPS1	Ap-CPS1-for1	ACATTGGCTTGTGTTGTCGC	*Hin*dIII	2
	Ap-CPS1-rev1	CACCGGATACACATTAGGAA		
CPS1	Ap-CPS1-for2	TTTTCTCCATCCTCCACTGC	-	1
	Ap-CPS1-rev2	TGCCTTTGTCGGTCCAGTAT		
IDH1	AP-IDH1-for	GCCTCAATATTTGCTTGGAC	-	1
	Ap-IDH1-rev	CATTGTTATCCAACTTGGCC		
IDH2	Ap-IDH2-for	GTCCAGAGTGATTTACTTGC	*Xba*I	1
	Ap-IDH2-rev	AGTAAAATTCCCGCGACACC		
IPI	Ap-IPI1-for	TATCGAAGAAAATGCACTCG	-	1
	Ap-IPI1-rev	GATGAACAGAAGGTAATCCA		
CAT	Ap-CAT1-for	CTCACCTGTGCTGATTTCCT		
	Ap-CAT1-rev	GGCCTCCAATGGAACTTAAC	-	2

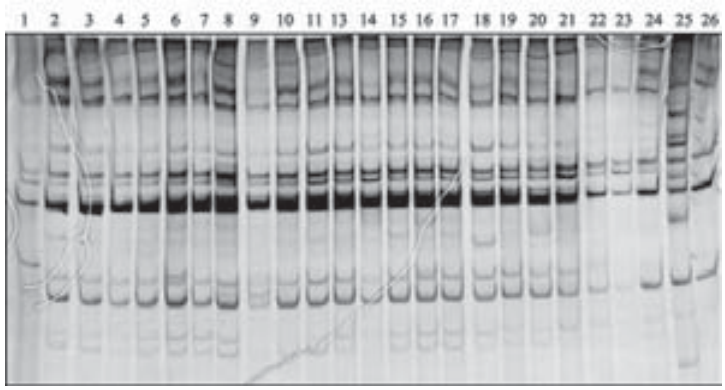

Figure 3 RAPD polymorphism amplified by primer OPA-16. Electrophoresis on 6% non-denaturing polyacrylamide gel. Lane 1– 25 = amplified fragment of *Andrographis* 25 accessions (as of Table 1) excepted No. 12, M = 100 bp DNA ladder (Fermentas).

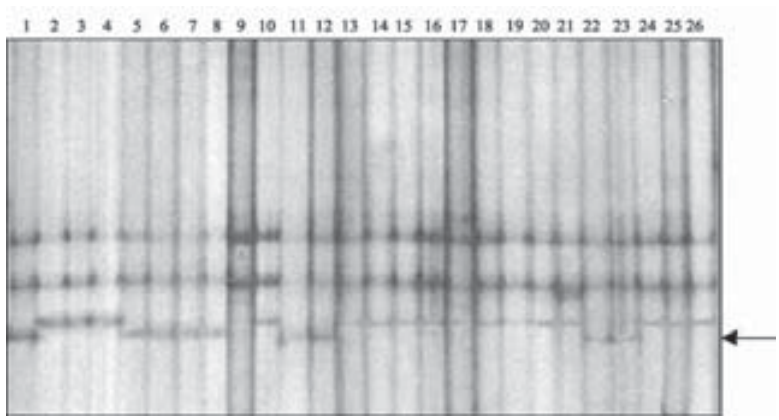

Figure 4 Polymorphism from SSCP amplified products among 26 accessions of *Andrographis* amplified by primer *CPS1-1* digested with *Hin*dIII.

Comparison of methods

SSCP markers detected less polymorphism than RAPD markers among 26 accessions. A possible explanation for the differences between SSCP and RAPD markers were that these two marker techniques targeted different portions of the genome. RAPD randomly detected the whole genome (Micheli *et al*., 1994; Powell *et al*., 1996) while SSCP specifically detected variations of secondary structures formed by single-stranded DNA of specific gene. SSCP technique revealed relatively narrow diversity of *Andrographis paniculata* in Thailand, but higher diversity by RAPD and morphological markers. SSCP markers from six specific primers were not appropriated for variability study on this plant species. Gene specific primers must contain regions where the sequence varies at high rate among populations. Therefore, *Andrographis paniculata* collections should be analysed with greater number of primers and more accessions from broader geographic range for duplicated genetic diversity estimation in this medicinal plant.

CONCLUSIONS

Genetic diversity of *Andrographis paniculata* based on 17 morphological characters and total lactone content showed the average distance coefficient of 3145.9 for all population and the total lactone content varied from 1.1-13.8%. RAPD technique revealed similarity coefficient of 67-95%, with the average for all population at 83% while only two loci showed polymorphic with SSCP marker.

RAPD and morphological character analysis showed higher diversity than SSCP analysis using specific primer. Neither morphological nor molecular markers were correlated with the accession collected site and the total lactone content.

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