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Evidence of molecular marker for genetic relationship of *Asystasia gangetica* (Linn) T. Anderson

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Asystasia gangetica (L.) T. Anderson (Acanthaceae) is widespread throughout the old world tropics. The plant is cultivated as an ornamental and various parts of the plant are also used traditionally for many ailments and diseases. Two subspecies are recognized in Thailand (1) A. gangetica micrantha that flowers are mostly white with two parallel purple blotches lines on the bottom petal lobe and (2) A. gangetica gangetica that flowers are various shades color as a pink, mauve or purplish with paler throats and white or cream with pale yellowish throats. However, there are comparatively a lesser number of reports focusing on intraspecific genetic diversity studies. The genetic diversity of a local subpopulation of A. gangetica was surveyed using random amplified polymorphic DNA (RAPD) analysis. A total of sixty-four random primers were used and eight primers (OPA-01, OPA-12, OPA-16, OPA-18, OPA-20, OPC02, OPC-05 and OPH-05) were produced reproducible fragments with easily recordable bands which were selected for analysis. A total of 91 bands were detected with an average of 11.4 bands per primer. The genetic similarities were estimated from banding pattern using UPGMA clustering method. The Pearson's similarity coefficients were used to construct a UPGMA dendrogram. Pair-wise estimates of genetic similarity ranged from 0.17 to 1.00. Five primary genotypes were found within a sampling of 30 individuals: white flowers with purple markings on the bottom petal lobe (A. gangetica micrantha), purple, white, pink and yellow flowers. Our result showed the RAPD marker for assessing genetic relationship among A. gangetica that correspond to flower color.

Key words: *Asystasia gangetica* (Linn) T. Anderson, Randomly Amplified Polymorphic DNA (RAPD), genetic relationship

Introduction

The family Acanthaceae consists of several important medicinal plants with broad array of biological activities. *Asystasia* is the genus of Acanthaceae, with approximately 70 species that are found in tropical and subtropical old

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world regions (Mabberley, 1987). *Asystasia gangetica* (Linn) T. Anderson known as Chinese violet (local common names: baya, yaya) which can grow and spread quickly. This species is dispersed by seeds and rhizomes that the nodes are easily develop roots and the seeds are dispersed from explosive capsules. It has green and oval-shaped leaves with rounded base occurring in opposite pairs. The multi-potential application of *Asystasia*, the leaves are eaten as a vegetable (Mepba *et al.*, 2007), traditional medicine and also used as an ornamental plant. *Asystasia* is known to have high nutritional value and various biologically active substances (Kanchanapoom and Ruchirawat, 2007; Hamid *et al.*, 2011; Tilloos *et al.*, 2012). Pharmacological studies, it has been claimed to have antiasthmatic, antidiabetic, anticancer and antioxidant, analgesic and antiinflammatory, antimicrobial and antifungal properties (Akah *et al*., 2003; Tilloos *et al*., 2012).

Two subspecies are recognized in Thailand (1) *A. gangetica micrantha* that flowers are mostly white in color with two parallel purple blotches lines on the bottom petal lobe and (2) *A. gangetica gangetica* that flowers are various shades color as a plum, purple, light pink, pink, white and yellow with paler to pale yellowish throats. However, genetic diversity had not been studied focusing on intraspecific in *Asystasia*. There is only a study about gametic chromosome number (n=13) and somatic chromosome number (2n=26) for *A. gangetica* in Singapore (Pandit *et al*., 2006).

Molecular techniques such as sequencing, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are common use for taxonomical, phylogenic and genetic diversity within and between populations. In recent years, the genetic relationship among Acanthaceae has been using sequence data from the trnL-trnF region in chloroplast DNA (Mcdade and Moody, 1999). However, RAPD marker is polymorphic and evenly distributed throughout the genome, simple and inexpensive. Therefore, this technique has been widely used in genetic diversity studies in different plant (Williams *et al*., 1990; Kumar, 1999). The genetic relationship in the Acanthaceae has been assessed using RAPD markers such as *Acanthus ilicifolius* Linn. (Lakshmi *et al*., 1997), *Andrographis paniculata* (Burm. f.) Nees (Lattoo *et al*., 2008) and *Clinacanthus nutan* (Fong *et al*., 2014). The main objective of this study was to characterize the extent and pattern of genetic diversity among a collection of *Asystasia* using RAPD marker which has been applied to increase our understanding of the distribution and extent of genetic variation within and between this species.

Materials and methods

Plant materials

A. gangetica was used in this study growing in Thailand. Plant samples were collected from 10 provinces during December 2013 to February 2014 and grown in experimental site. A detailed description of this plant is given in Table 1. Thirty plants were chosen according to their flower pattern and color. After acclimatization 2-3 young leaves were harvested fresh for DNA isolation. Six of these trees have white flowers with purple markings on the bottom petal lobe and twenty-two have various shades color with paler to pale yellowish throats (3 plum, 3 purple, 6 white, 3 light pink, 4 pink and 5 yellow flowers) that shown in figure 1.

Table 1. Numbers of *A. gangetica* from 10 provinces has been used in the study

Numbers	Provinces	Number of samples
1	Bangkok	10
2	Ratchaburi	5
3	Chachoengsao	1
4	Srisages	1
5	Khonkaen	4
6	Prachinburi	1
7	Nonthaburi	4
8	Phayao	2
9	Samutsakhon	1
10	Pathumthani	1

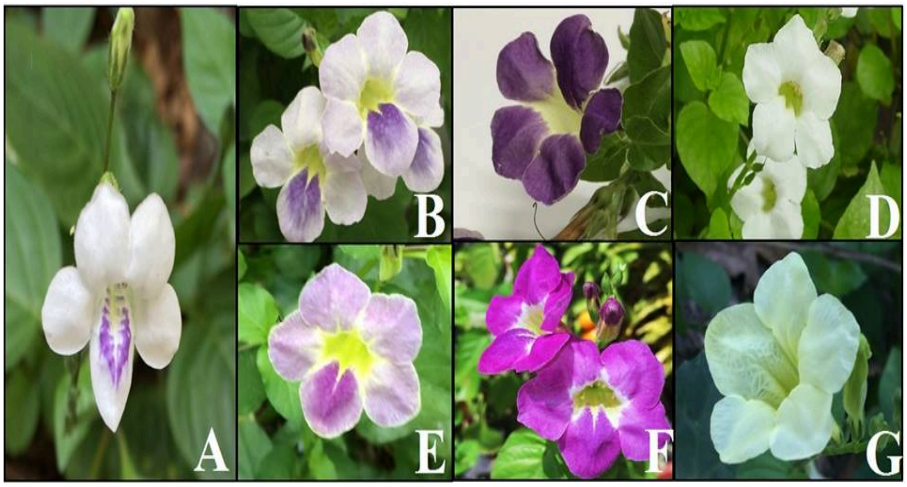

Fig 1. Flower pattern and color in *Asystasia gangetica* (Linn) T. Anderson. A: white flower with purple markings on the bottom petal lobe, B: plum, C: purple, D: white, E: light pink, F: pink and G: yellow flower color

DNA extraction

For each sample, total genomic DNA was extracted from fresh leaves by a slightly modified CTAB (Cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987). One hundred gram of fresh leaves were frozen in liquid nitrogen and ground into a fine powder. 2XCTAB extraction buffer [2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4M NaCl and 1% PVP] with β-mercapthoethanol was added and the mix was incubated at 65°C for 45-60 min. Equal volume of chloroform: isoamyl alcohol (24: 1) was carried out and the extract was centrifuged at 14,000 rpm/min for 5 min. After centrifugation, the supernatant was incubated with two µL of RNaseA (20 mg/mL) at 37°C for 1 hour. 10% CTAB buffer and chloroform: isoamyl alcohol (24: 1) was added and the mixture was centrifuged at 14,000 rpm/min for 5 min. Two-third volume of isopropanol was added to the supernatant and incubated at -20°C for 1 hour. DNA was collected by centrifugation at 14,000 rpm/min for 20 min and washed with 70% (v/v) ethanol. After centrifugation, the DNA was dehydrated with absolute ethanol and incubated at 37°C for 10 min. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Then, DNA was quantified and analyzed for quality by a spectrophotometry and agarose electrophoresis method on 1 % agarose gel in 1X TBE buffer.

RAPD analysis

RAPD analysis of genomic DNA was done using 10-mer random oligonucleotide primers (William *et al*., 1990). The extracted genomic DNA in each sample was diluted with TE buffer to a concentration of 100 ng/ µl. The twenty microliter of mixture was consisted of 100 ng of genomic DNA, 1X PCR buffer, 1.25-2 mM $MgCl_2$, 0.2 mM dNTPs, 1 pmol RAPD primer, 1 unit of *Taq* DNA polymerase and deionized water. Amplification was done using thermal cycler with following program: initial denaturation at 94°C for 1 min 45 sec, followed by 10 cycles of 1 min at 94°C, 1 min at 37°C and 1 min 45 sec at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min 45 sec at 72°C and final extension at 72°C for 7 min 30 sec. RAPD products were separated by agarose electrophoresis on 1.5 % agarose gel in 1X TBE buffer along with 100 bp plus DNA ladder (vivantis). The gels were stained with 10 µg/ml ethidium bromide solution, examined under UV light transilluminator and photographed using gel documentation system.

Data analysis

In data scoring and analysis of RAPD: Clear and well resolved bands were compared with each other and DNA fragments were scored as a binary character (1 for presence and 0 for absence) from each primer. Coefficients of genetic similarity (GS) between the individuals were obtained using the simple matching coefficient (Rohlf, 2000) and constructed a dendrogram based on genetic similarity matrix using the UPGMA (unweighted pair group mean average) method (Sokal and Sneath, 1973) by NTSys Version 2.0e program. Including, principle coordinate analysis (PCA) among all individuals was analyzed using SPSS software.

Results and discussion

Sixty-four decamer primers were used for preliminary screening. Only eight primers (12.5%) showed reproducible fragments with easily recordable bands and gave polymorphisms. These distinguish banding patterns can be successfully and eight RAPD primers could amplify DNA from all *A. gangetica* samples. The number of RAPD bands per primer ranged from 9 (OPA-12) to 15 (OPA-18) with an average 11.4. The amplified products were then categorized based on their size ranging from 300 to 3,000 bp. In total 81 polymorphic bands were scored which OPA-18 and OPA-20 primers gave the highest number of polymorphic fragments. The 89.00 percentages of polymorphic bands were

given from all of RAPD primer. A summary of eight primers sequence, amplified products and percentages of polymorphic bands from this study is shown in Table 2. Beside, the RAPD profiles generated by the primer OPA-16 are shown in Figure 2.

Table 2. Primer codes, sequences, number of amplified bands and polymorphism detected by the use of eight RAPD primers in 30 samples of *A. gangetica*

Primer codes	5'-sequence-3'	Number of amplified bands	Number of polymorphic bands	Polymorphic bands (%)
OPA-01	3' CAGGCCCTTC 5'	10	9	90.00
OPA-12	3' TCGGCGATAG 5'	9	6	66.67
OPA-16	3' AGCCAGCGAA 5'	10	9	90.00
OPA-18	3' AGGTGACCGT 5'	15	13	86.67
OPA-20	3' GTTGCGATCC 5'	14	13	92.86
OPC-02	3' GTGAGGCGTC 5'	12	10	83.33
OPC-05	3' GATGACCGCC 5'	11	11	100.00
OPH-05	3' AGTCGTCCCC 5'	10	10	100.00
	Total bands	91	81	89.00

The RAPD marker revealed by eight pairs of selected primers was used for the cluster analysis. Dendrogram constructed with the unweighted pair-group method with arithmetic mean (UPGMA) of genetic relationships among 30 samples based on simple matching coefficients varied from 0.55 to 0.95 (Figure 3) based on RAPD polymorphic data using the SIMQUAL program in the NTSYSpc software. The highest similarity was detected between the purple flowers (BY07 and BY11) and the lowest similarity was observed between BY04 and BY07/11. The thirty samples were grouped into three clusters at a 55% similarity level. The first major cluster (A) consisted of 6 samples (BY02, 04, 20, 25, 26 and 30). This cluster is belonging to *A. gangetica micrantha* that flowers are mostly white in color with two parallel purple blotches lines on the bottom petal lobe. The second major cluster was formed by 12 samples consisting of plum (B), purple (C) and white (D) flowers. The last major cluster was formed by 12 samples consisting of pink (E) and yellow (F) flowers. This resulted suggests that three subspecies will be recognized (1) *A. gangetica micrantha*, (2) the flowers are shades color as a plum, purple and white flowers

with paler yellowish throats and (3) the flowers are shades color as pink and yellow with pale yellowish throats.

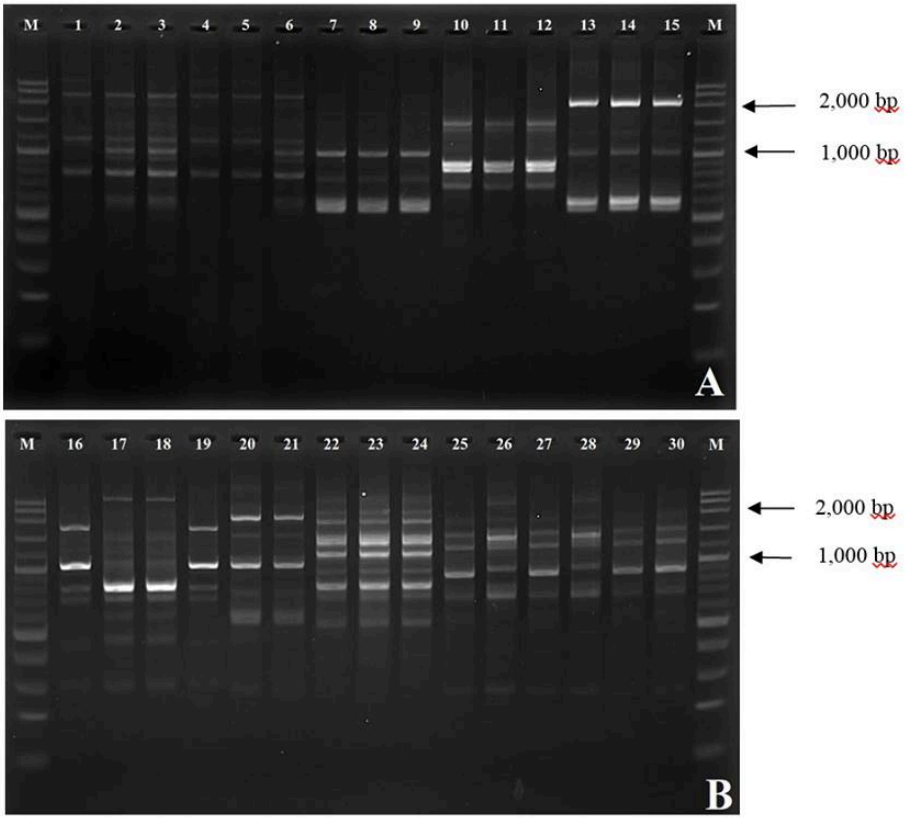

Fig. 2. RAPD profile of 30 samples of *A. gangetica* obtained with primers OPA-16, A: lanes 1-15 and B: lanes 16-30 represent samples 1-30 and M: markers (100 bp plus DNA ladder)

However, the thirty samples were grouped into six different genotypes at a 78% similarity level (Figure 3). The first genotype (A) only consisted of *A. gangetica micrantha*. Groups B, C and D were formed by 3 plum, 3 purple and 6 white flowers, respectively. Including, E and F were formed by pink and yellow flowers, respectively. This result showed the RAPD marker for assessing genetic relationship among *A. gangetica* that related with flower color.

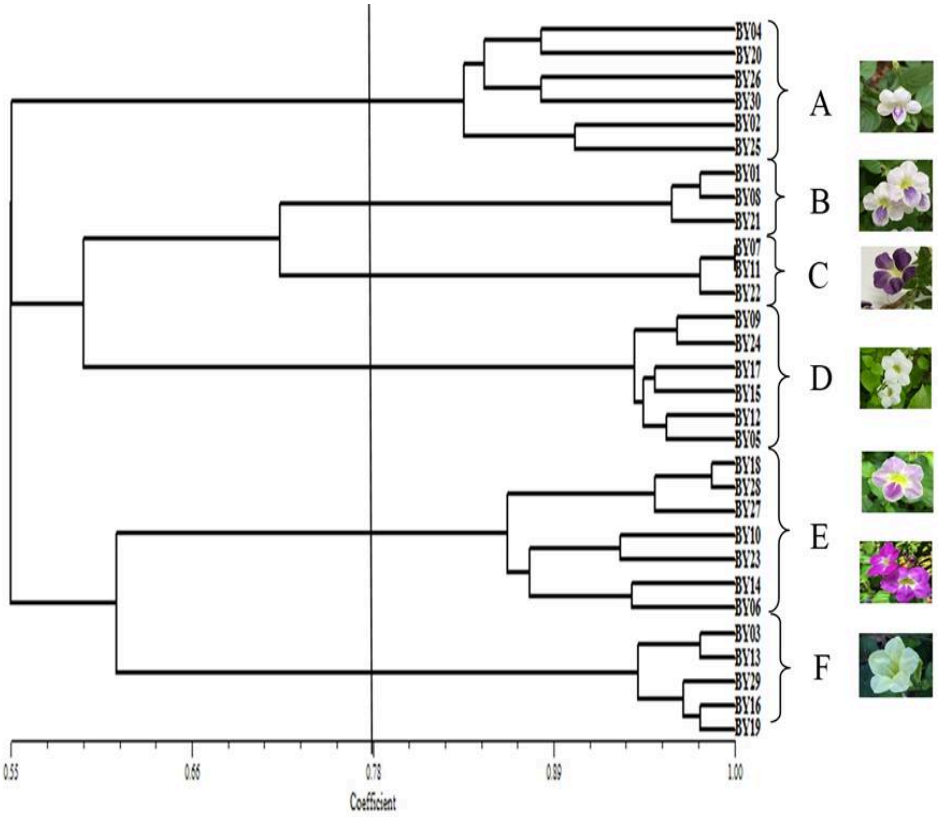

Fig 3. Dendrogram constructed with the unweighted pair-group method with arithmetic mean (UPGMA) of genetic relationships among 30 samples of *A. gangetica* based on simple matching coefficients from RAPD marker: A-F represent the six different clusters at a 78% similarity level

Conclusion

The molecular marker techniques not have been currently available for the analysis of genetic relationship in *A. gangetica*. This is first report using molecular marker which can be employed successfully for assessment of the genetic relationship of *A. gangetica* collected from different regions of Thailand. Dendrogram constructed with the UPGMA among 30 samples based on simple matching coefficients. In the present study was showed the high level of genetic diversity. Six primary genotypes were found within samplings that correspond

to flower color. However, genetic relationships of *A. gangetica* will be studied using other molecular markers to support this study.

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