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Full Length Research Paper

Genetic diversity and inbreeder specie of *Andrographis paniculata* (Burm. f.) Nees by randomly amplified polymorphic deoxyribonucleic acid (RAPD) and floral architecture analysis

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"Fha-Tha-Laai-Joan" (*Andrographis paniculata* (Burm. f.) Nees) is a medicinal plant that is, widely used in Thailand and some other countries for therapeutic value, 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* which were 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 8.25 bands per primer pair. The phylogenetic tree derived from RAPD data showed that *A. paniculata* were divided into only one group in which the high similarity values were between 0.81-1.00. The results obtained 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. The floral architecture and reproductive habitat study of *A. paniculata* revealed the same results as of RAPD markers analysis. This species is thus hermaphroditic and acts as habitual inbreeder.

Key words: *Andrographis paniculata,* randomly amplified polymorphic DNA (RAPD), floral architecture, monomorphic, Thailand.

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 research found various pharmacological activities of *A. paniculata*, including immune-stimulatory (Puri et al., 1993), anti-Human immunodeficiency virus (HIV) (Otake et al., 1995; Calabrese et al., 2000), hepatoprotective (Handa and Sharma, 1990; Kapil et al., 1993), anti-malarial (Misra et al., 1992) and cardiovascular (Zhang and Tan, 1997; Zhang et al., 1998). A major problem for the use of *A. paniculata* for judicious selection in crop improvement program is the lack of genetic background data. As the accessions are poorly characterized, it is important at the beginning of breeding program to discriminate among

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

available genotypes to establish the level of genetic diversity and thereby identifying the most suitable materials for crossing. Genetic diversity among individuals or populations can be determined using morphological and molecular markers.

In contrast, molecular markers based on deoxyribonucleic acid (DNA) sequence polymorphism, are independent of environmental conditions. Molecular markers provide a quick and reliable method for estimating genetic relationships among genotypes of any organism (Thormann et al., 1994). Among the different types of molecular markers available, The RAPD are widely used for the assessment of genetic diversity because of their simplicity, fast and easy to perform and comparatively cheaper, requiring no prior knowledge of 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; Welsh et al., 1991). The RAPD-based molecular markers have been found to be useful in *A. paniculata* (Padmesh et al., 1998; Maison et al., 2005; Latto et al., 2006; Kumar and Shekhawat, 2009). Inspite of the immense therapeutic value, lack of genetic information in Thai *A. paniculata* has lead to sequential problem for the selection of divergent genotypes for crossing, as well as effective conservation and management of its germplasm resources. Consequently, the present study was performed to determine genetic diversity of *A. paniculata* accessions collected from different locations using RAPD markers and floral architecture analysis. This investigation will be insightful for further studies on germplasm and also for generating a database on *A. paniculata* which can be used for breeding program and bioactive compound study.

MATERIALS AND METHODS

Plant materials

Fifty-eight accessions of *A. paniculata* were collected from five populations of geographic locations in 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 of Thailand (Table 1).

DNA extraction

Total genomic DNA from the young leaves of *A. paniculata* was isolated using Cetyltrimethylammonium bromide (CTAB) modified protocol (Murray and Thompson, 1980) at DNA Technology Laboratory, Kamphangsaen Campus, Kasetsart University. 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 ul of pre-heated extraction buffer (20 mM ethylene diamine tetraacetic acid (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 ∝l of chloroform: isoamyl alcohol (24: 1, v/v) and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube. The DNA was precipitated by adding a two-third 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 DNA pellet was dried by leaving the tubes uncovered at 37°C, 20 to 30 min. The DNA was dissolved in 150 ∝l TE buffer (10 mM Tris and 1.0 mM EDTA, pH 8). To confirm the concentration and quality of the DNA samples, a 2 ∝l of the stock DNA solution was run in 0.8% agarose gel stained in 1 ∝g/ml of ethidium bromide solution and compared visually in Lambda DNA standards of known concentrations under ultraviolet (UV) illumination. An aliquot of the isolated DNA for each sample was diluted to 20 ng/∝l in TE buffer and stored at 4°C for use in subsequent assays, while the stock of DNA samples were stored at -40°C.

DNA analysis by RAPD marker

The RAPD amplifications were carried out using thirty random primers (Operon Technologies, Alameda, CA, USA). RAPD reactions were done in a total volume of 15 ∝l containing 200 ∝M of deoxynucleotide triphosphates (dNTPs), 2.5 mM $MgCl_2$, 0.3 U Taq DNA polymerase, 5 pmol of each primer and 50 ng of DNA Template. DNA amplification was performed in a DNA Thermal Cycler 600 (Applied Biosystems) in 0.5 ml Polymerase chain reaction (PCR) tubes programmed for initial denaturation at 93°C for 1 min, 1 min at 35°C and 2 min at 72°C, followed by 44 cycles for 1 min at 95°C, at annealing temperature of 35°C for 1 min and at 72°C for 2 min as an extension step. The final extension step was done for 10 min at 72°C and the reactions were kept at soak file at 4°C. Amplification products were subsequently separated on 15% agarose gel with ethidium bromide staining.

Data analysis

The RAPD profiles generated were score as discrete variables using 1 to indicate presence and 0 to indicate 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

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

Region	No of accession	Origin of geographical distribution (Province)
North	**8**	Chiang Mai (APCM1, APCM2) Chiang Rai (APCR1, APCR2) Uttaradit (APUT) Lampang (APLP) Phayao (APPY) Sukhothai (APST1)
Northeast	24	Nakhon 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) Prachuab (APPC1, APPC2) Phetchaburi (APPB) Prachin Buri (APPR1, APPR2) Sing Buri (APSB) Saraburi (APSA)
East	4	Trat (APTD) Chanthaburi (APCT) Rayong (APRA1, APRA2)
South	11	Chumphon (APCP) Satun (APST) Songkla (APSO1, APSO2) Phatthalung (APPL) Trang (APTr) Krabi (APKB1, APKB2) Phangnga (APPN) Nakhon Si Thammarat (APNS) Surat Thani (APSRT)
Total	58	

Table 2. RAPD primers used for *A. paniculata* identification by RAPD, the number of bands produced and Polymorphic bands percentage.

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

(Sneath and Sokal, 1973). Genetic similarity among all accessions was calculated using the NTSYS-pc programme version 2.01e (Rohlf, 1990).

Floral architecture analysis

For investigating the *A. paniculata* floral architecture, bearing flower buds were collected in each developmental stage. The four stages of inflorescence were kept and their bracts were separated to obtain internal reproductive organs. To check the extent of self pollination behavior, the internal reproductive organs of four inflorescence stages were examined and photographed using a fluorescence light microscope.

RESULTS AND DISCUSSION

For preliminary screening, thirty primers were used and it was found that only eight primers with 60% of GC content could successfully amplify all of 58 *A. paniculata* genomic DNA samples.

Level of polymorphism

A total of 66 bands were amplified by the eight oligonucleotide primers used in the RAPD analysis. All of the eight 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* accessions was very low. The average 37.63% of polymorphic bands were given from all of RAPD primer (Table 2). The RAPD profiles generated by the primer OPZ-04 were shown in Figure 2. The amplified products were then categorized based on their size

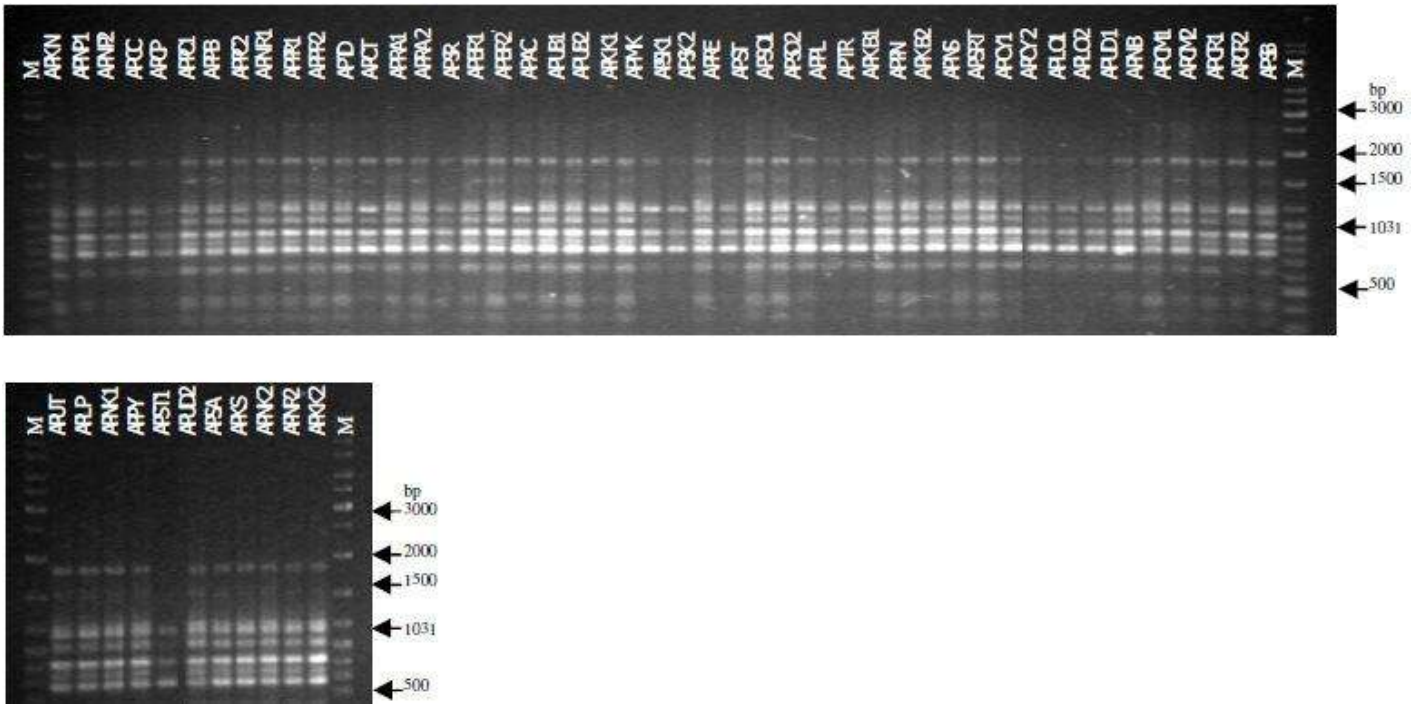

Figure 2. Monomorphic pattern of RAPD profile from the genomic DNA of *A. paniculata* generated by primers OPZ-04. Lane M, molecular marker (GeneRuler ^{TM} DNA Ladder Mix).

ranging from 500 to 1,900 bp and the numbers of bands produced ranged from 3 using 0PZ-08 to 12 using OPW-05 primer (Table 2). Pair wise comparisons among all accessions were made and genetic similarity values were estimated based on Nei's method (Nei and Li, 1979). The mean of genetic similarity for all accession was high at 0.905 with variation ranging from 0.809 to 1.000. It is interesting to note that of the eight primers employed, nearly most showed monomorphic fragments of loci as shown in Figure 2.

The data obtained from a genetic similarity values were then used to determine the genetic relationship of these *A. paniculata.* The UPGMA cluster analysis based on the Jaccard's coefficient was performed and used to construct the phylogenetic tree and showed as a dendrogram in Figure 3. All accessions of *A. paniculata* collected from five populations of geographic locations in Thailand were grouped as one cluster at an arbitrary cut-off at 87% similarity level on the dendrogram. From cluster analysis, the investigation of genetic variation in *A. paniculata* in this study using RAPD markers revealed that almost all of the samples collected from various geographic locations of Thailand did not very in genetic pattern. The RAPD marker analysis demonstrated a clear lack of differentiation based on these grouping in Thailand. The result 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 rang. 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 plants in many parts of Asia (Widen and Swenson, 1992). This result further supported the hypothesis that *A. paniculata* is an inbreeder species. The result in this study corresponded to that of Padmesh et al. (1998) which reported that *A. paniculata* from Thailand showed a different genotype from other accessions suggesting that it is geographically distant from the rest. The result in this study corresponded to that of Padmesh et al. (1998) which reported that *A. paniculata* samples from Thailand were closely related to those from India.

This implies that *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. However, this study revealed very low level of genetic variability detected across all accessions using three marker systems. This result also corresponded to the report of Maison et al. (2005) that the genetic similarity among 25 Thai *A. paniculata* accessions using RAPD was more than 67% and average similarity coefficient for all population was 0.83, indicating a high genetic relatedness. In addition, single-strand conformation polymorphism (SSCP) analysis showed little polymorphism of specific amplified products existing among these accessions. Furthermore, the similar results using molecular marker reported by Sakuanrungsirikul et al. (2008) showed no genetic variation among *A. paniculata* accessions from nine distribution areas

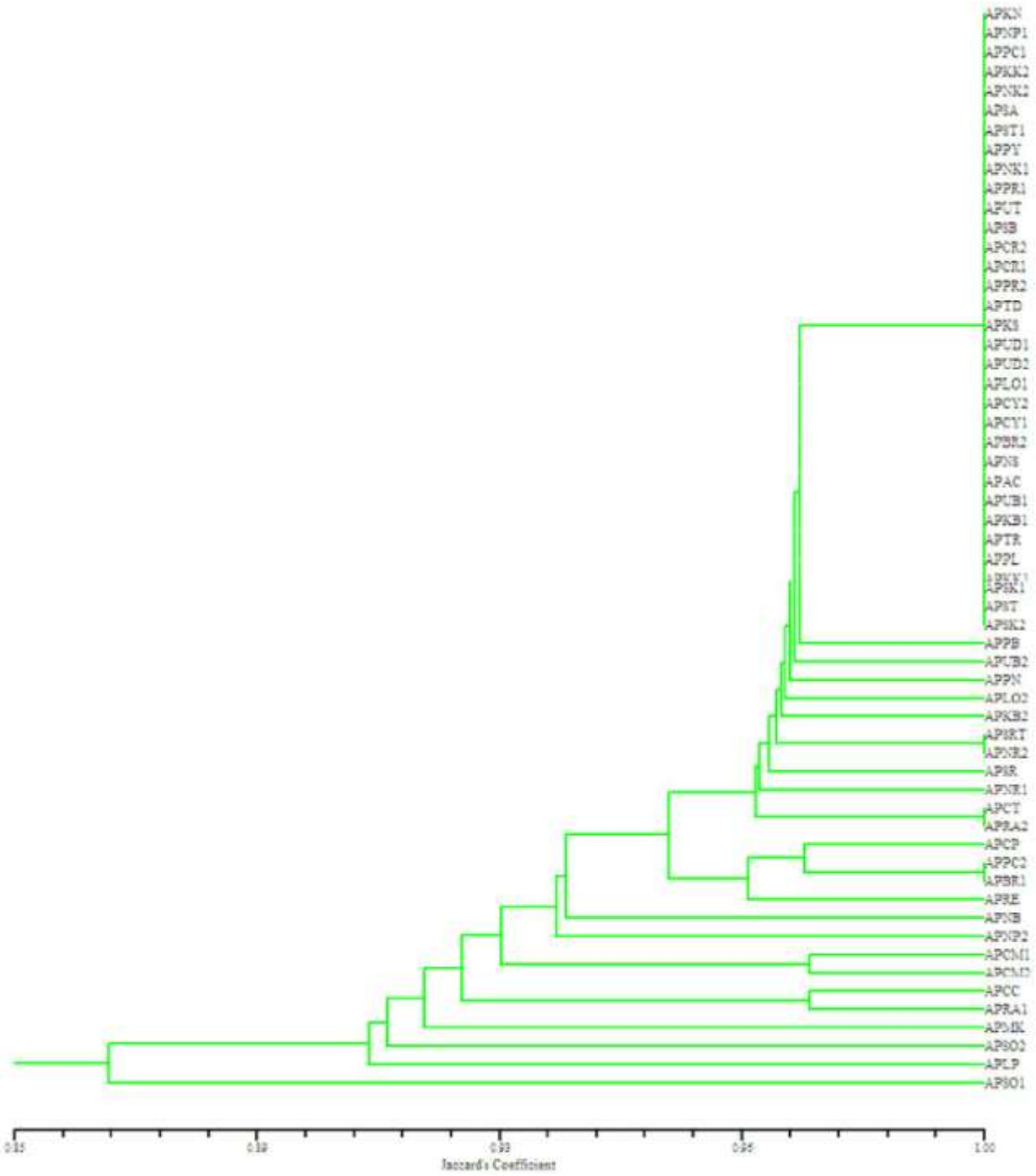

Figure 3. Dendrogram showing the genetic similarity among 58 *A. paniculata* accession as derived from RAPD data using the unweighted pair group method of arithmetic means (UPGMA).

throughout Thailand. It was reported that all loci generated by inter simple sequence repeat. (ISSR)-Touchdown PCR and high annealing temperature RAPD techniques were found to be monomorphic across all accessions. The result in this study also corresponded with Latto et al. (2006) and Kumar and Shekhawat, (2009) which reported the 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 is in conformity with breeding behavior of the species. As previously reported for genetic variability analysis

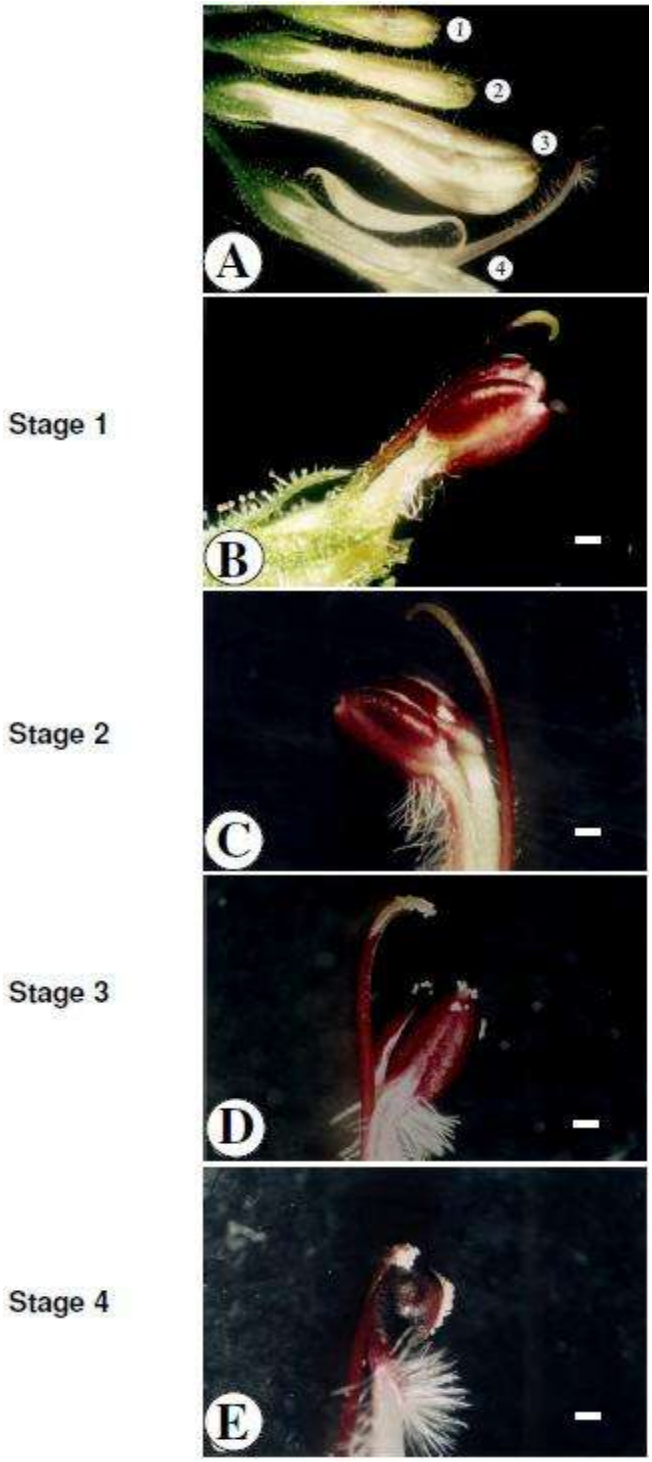

Figure 4. Reproductive organs of *A. paniculata* flower (A) Stage 1 to 4 of flower (B)-(E) inside of flower shown clogged stigma and dehiscent anther in stage 1 to 4, respectively (bar = 100 µm).

including this study, all of DNA-based molecular markers techniques (RAPD, SSCP and ISSR markers) have been found similarly proven in *A. paniculata*.

It has been well explained that genetic variation of *A. paniculata* accessions could not be detected. It is unlikely that these species are the genetic variations considering that they are hermaphroditic, self-compatible and habitual inbreeders. Intimate proximity of adpressed stigma with 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). This present study showed that all of 58 collected samples of *A. paniculata* revealed extremely high genetic similarity. Although some amount of variability was detected with RAPD markers, it did not contribute to 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 high level of genetic similarity in this study indicated that RAPD markers can be used to evaluate their genetic structure. The result also revealed the extreme presence of single varieties of *A. paniculata* in Thailand.

Conformation of the DNA marker results using floral architecture study

Results of the monitoring in floral architecture of *A. paniculata* using stereomicroscope indicated that the plant is in conformity with DNA fingerprinting patterns revealed by Simple Sequence Repeat (SSR), amplified fragment length polymorphism (AFLP) and RAPD markers. *A. paniculata* is hermaphroditic, self compatible and its flower structure showed a habitual inbreeding behavior. Intimate proximity of adpressed stigma with the anthers (Figures 4 and 5) and synchronization of anther dehiscence and stigma receptivity allow for obligate autonomous self pollination in the species. These RAPD marker result confirmed that the reproductive habitat of *A. paniculata* acts as a habitual inbreeder specie. The very low level of genetic differentiation among the 58 germplasm accessions from different localities in Thailand suggests that they all share the same variety. Floral architecture of this specie revealed some organs intimate self-compatibility pollination due to proximity of adpressed stigma with the anthers. In the bud stage, pollen grains are dehisced itself before flower opening and synchronization of anther dehiscence and receptivity of stigma, providing for obligate autonomous self pollination in the species. A clogged stigma and dehiscent anthers were found in the bud stage and a mass of pollen grains were seen on the surface of anthers. Stigma also remains close to anthers. Even though the flowers of *A. paniculata* were frequently visited by the honeybee, the visitors were often found to carry pollen mass away from the flowers. However, these RAPD marker results remain a mutual same pattern. Although cross pollination by visitors could not be entirely eliminated, it is certain to explain that *A. paniculata* is

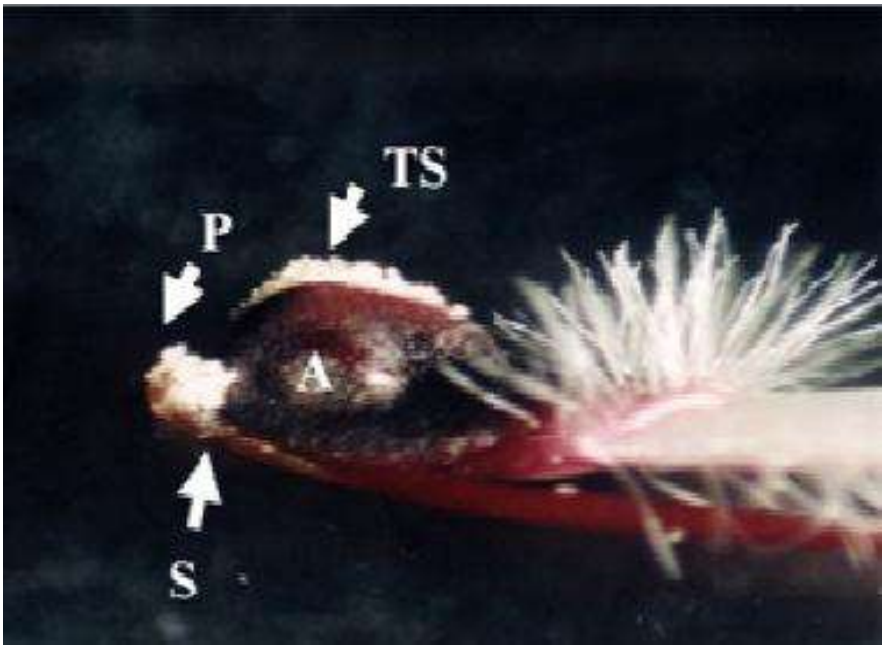

Figure 5. Plant architecture: Pollen (P); clogged stigma (S) adpressed to transversely (TS) dehiscent anthers (A) provides for obligate autonomous selfing (bar = 100 µm).

essentially self pollinated even before flower opening; and subsequent visits of the insects are mostly directed towards benefiting the visitors.

Conclusions

Genetic diversity of *A. paniculata* based on RAPD markers analysis showed the high similarity coefficient of 81 to 100%. This study has also demonstrated that the RAPD marker is a promise approach for evaluating genetic diversity of *A. paniculata*, which revealed that all the 58 collected samples from various locations of Thailand are likely to belong to the same variety. Their genetic are relatively undifferentiated across a large geographic range. Furthermore, the floral architecture and reproductive habitat study of *A. paniculata* revealed the same results as RAPD markers analysis. This species is thus hermaphroditic and acts as habitual inbreeder. Further research is needed to induce genetic variability of *A. paniculata* in our germplasm by mutation process to access its genetic background change. Further analysis is also needed on the sequencing of these mutated genes which could be used in the breeding program for optimizing its genetic amelioration and enriching its bioactive compound.

Abbreviations: RAPD, Randomly amplified polymorphic deoxyribonucleic acid; **TTM,** Thai traditional medicine; **HIV,** human immunodeficiency virus; **CTAB,** cetyltrimethyl ammonium bromide; **EDTA,** ethylene diamine tetraacetic acid; **UV,** ultraviolet; **PCR,** polymerase chain reaction; **UPGMA,** unweighted pair-group method with arithmetic averages; **SSCP,** single-strand conformation polymorphism; **SSR,** simple sequence repeat; **AFLP,** amplified fragment length polymorphism; **dNTPs,** deoxynucleotide triphosphates; **ISSR,** inter simple sequence repeat.

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