บทความวิจัย

การตรวจสอบความหลากหลายทางพันธุกรรมของ *Stachytarpheta jamaicensis* (L.) Vahl โดยใช้เทคนิค High Annealing Temperature-RAPD (HAT-RAPD)

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บทคัดย่อ

การตรวจสอบรูปแบบพันธุกรรมของ *Stachytarpheta jamaicensis* ด้วยเทคนิค HAT-RAPD ให้ความหลากหลายของแถบดีเอ็นเอ (polymorphic band) จำนวน 17 แถบ ซึ่งมีขนาด 300 ถึง 1,500 bp และให้รูปแบบ haplotype จำนวน 19 แบบ เมื่อวิเคราะห์ความหลากหลายทางพันธุกรรมของ *S. jamaicensis* พบว่า กลุ่มประชากร *S. jamaicensis* ในจังหวัดนครนายก มีค่า % polymorphic band และ expected heterozygosity มากที่สุด (P = 64.71% และ H_e = 0.224) รองลงมากลุ่มประชากรในจังหวัดสระแก้ว (P = 29.41% และ H_e = 0.080) และกลุ่มประชากรในจังหวัดหนองคาย (P = 17.65% และ H_e = 0.066) นอกจากนั้นสายสัมพันธ์วิวัฒนาการ Neighbor-joining trees ที่วิเคราะห์จาก Nei's genetic distance สามารถแบ่ง *S. jamaicensis* เป็น 2 กลุ่ม ได้แก่ กลุ่มประชากร ในจังหวัดนครนายกและปทุมธานี และกลุ่มประชากรในจังหวัดสระแก้วและหนองคาย ผลการวิเคราะห์ AMOVA ยืนยันการมีโครงสร้างพันธุกรรมภายในชนิด *S. jamaicensis* ที่ใช้ในการศึกษา และยังแสดงความแตกต่างทางพันธุกรรมระหว่างกลุ่มประชากรในจังหวัดนครนายกและปทุมธานี และกลุ่มประชากรในจังหวัดสระแก้วและหนองคาย (ϕ_{RT} = 0.248) ความแตกต่างระหว่างประชากรทั้ง 4 กลุ่ม (ϕ_{PR} = 0.493) และระหว่างตัวอย่างภายในกลุ่มประชากร (ϕ_{PT} = 0.619)

คำสำคัญ: ความหลากหลายทางพันธุกรรม *Stachytarpheta jamaicensis* (L.) Vahl HAT-RAPD

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Genetic Polymorphisms of *Stachytarpheta jamaicensis* (L.) Vahl based on a High Annealing Temperature RAPD (HAT-RAPD)

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ABSTRACT

The genetic patterns of *Stachytarpheta jamaicensis* were determined by using HAT-RAPD markers. Seventeen polymorphic HAT-RAPD markers showed a length from 300 to 1,500 bp, and nineteen haplotypes were obtained. The genetic diversity of *S. jamaicensis* was indicated by the percentages of polymorphic bands and the values of expected heterozygosity showing the greatest in Nakorn Nayok populations (P = 64.71% and H_e = 0.224), and the lowest in other populations (P = 11.76% and H_e = 0.048 for Pathum Thani, P = 29.41% and H_e = 0.080 for Srakaew, P = 17.65%, and H_e = 0.066 for Nong Khai). The Neighbor-joining tree from Nei's genetic distances demonstrated two groups of *S. jamaicensis,* one consisted of Nakorn Nayok and Pathum Thani populations and other consisted of Srakaew and Nong Khai populations. The AMOVA result confirmed the existence of intra-specific genetic structure in *S. jamaicensis* populations used in this study. Moreover, the genetic differentiations were significantly found between the two geographic regions: Nakorn Nayok and Pathum Thani group and Srakaew and Nong Khai group (ϕ_{RT} = 0.248), among populations (ϕ_{PR} = 0.493) and among individuals within populations (ϕ_{PT} = 0.619).

Keywords: Genetic polymorphisms *Stachytarpheta jamaicensis* (L.) Vahl HAT-RAPD

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Introduction

The genus *Stachytarpheta* is a weedy plant (Verbenaceae family) which is widespread through tropical and subtropical America, and a few species are found in tropical Asia, Africa, and Oceania. Among the species, *Stachytarpheta jamaicensis* Linn Vahl is a weedy annual or small shrub 60-120 cm long and has branches. It consists of purple flowers and simple leaves with coarse teeth along the edges [2-3]. *S. jamaicensis* has been introduced in various countries including Nigeria, Brazil, Taiwan and Thailand [7, 12, 22]. This plant well all over years and has been used as a traditional folk medicine for antifever, antidiarrheal, liver treatment, suppressing cough, lowers blood pressure, stimulates digestion, heals wounds, mildly laxative, anti-inflammatory and abortifacient [11, 13, 15]. Recently, the leaves of *S. jamaicensis* were popular to use as an herb drink in developing countries (e.g. Nigeria, Taiwan) [7, 8]. The major bioactive compounds (e.g. flavones and flavonoids, terpenes, phenols, quinones and steroids) for this plant species have been reported by Duke (1992) [4].

Nowadays, DNA molecular techniques such as randomly amplifed polymorphic DNA (RAPD) have been applied for all parts of several organisms in order to identify genetic data without influence of environment and external morphology of living [18]. The RAPD method is one of the popular molecular markers using the principle of polymerase chain reaction (PCR) with single short arbitrary primers to detect genetic polymorphisms. This technique is not required any information from DNA sequence, easy to use, low cost and fast [20, 21]. However, it showed disadvantages in reproducibility and reliability because of a low annealing temperature used [1]. Recently, a high annealing temperature RAPD (HAT-RAPD), reported by Eimert et al. (2003) [5] are popular, since it showed more reproducibility and polymorphism by increasing the annealing temperature between 45-62°C. This technique has been used for detecting molecular phylogeny in several plant species such as banana [16]. Although *Stachytarpheta* sp. is found in some regions of Thailand such as Hui Kvang, Kanchanaburi province [10], the major information about geographic distribution, genetic diversity for this plant present in this country has never been reported until now.

Our major goal of this study was to investigate genetic polymorphisms of *S. jamaicensis* presented different regions in Thailand by using the HAT-RAPD technique. The basic knowledge obtained in this study may useful for management of genetic conservations of this plant, and may apply for folk remedies in several urban communities of Thailand.

Materials and Methods

Plant materials

Thirty nine samples of *S. jamaicensis* were collected from Nakorn Nayok (N=12), Pathum Thani (N=9), Srakaew (N=13) and Nong Khai (N=5) provinces of Thailand between January and September 2013 (Figure 1A). They were basically identified by using their external morphology [9] as shown in Figure 1.

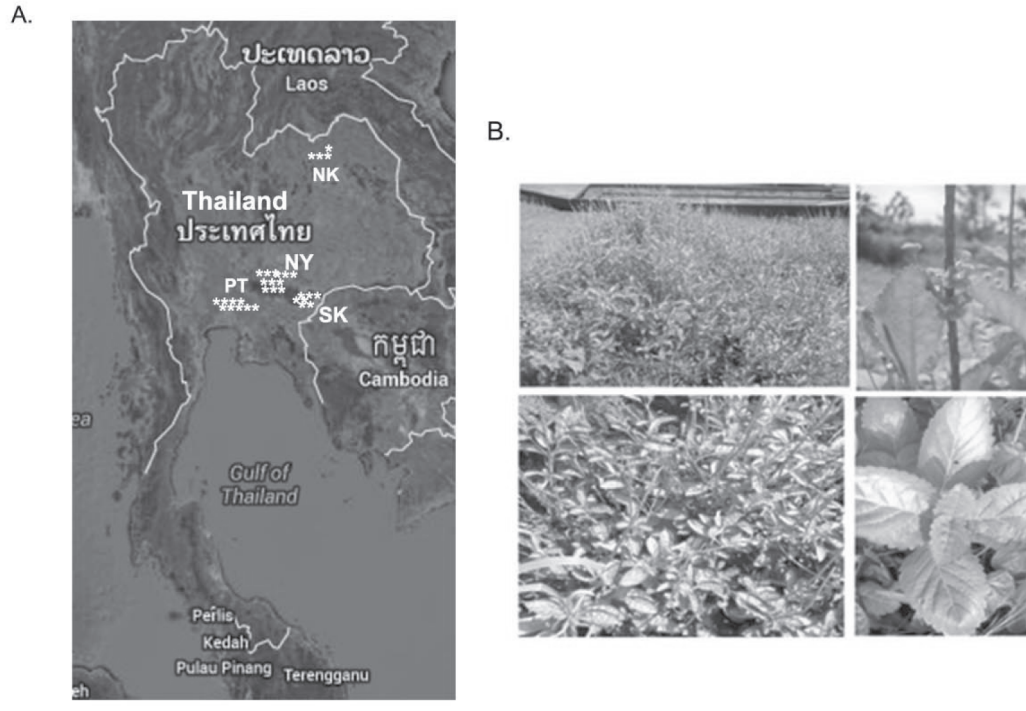

Figure 1. Sampling regions of *S. jamaicensis.* A showed four sampling areas of Thailand; PT = Pathum Thani, NY = Nakorn Nayok, SK = Srakaew and NK = Nong Khai, shown in Google Earth version 7.1.2.2041 and B are samples of *S. jamaicensis.*

Genomic DNA isolations

Genomic DNA of fresh leave samples was extracted according to the method of Plant genomics DNA extraction Mini Kit (RBC Bioscience, Taiwan). Briefly, the leave tissue of *S. jamaicensis* (50 mg) was cleaned with distilled water and grinded with sterile pestle. The sample was transferred to new microcentrifuge tube, and added with 400 µl GP1 buffer and 5 µl RNase A (10 mg/ml), mixed and incubated at 65°C for 10 min. After that, A 100 µl GP2 buffer were then added and incubated on ice for 3 min. The supernatant were obtained by using a centrifugation at 13,000 rpm for 3 min, and added with GP3 buffer for 1.5 time volume, then apply the sample to a GD column placed into a 2 ml collection tube and centrifuge (13,000 rpm) for 3 min. After that, GD column was applied with W1 buffer, centrifuged and discarded flow-through in collection tube, and then applied 600 µl of Wash buffer to the GD column, centrifuged and discarded flow-through in collection tube. Finally, dried GD column was added with 100 µl of Elution buffer and centrifuged 13,000 rpm for 3 sec. Then, DNA isolated from the samples was verified on 0.7% agarose gel electrophoresis by staining with ethidium bromide, and exposed to UV light.

HAT-RAPD analysis

The DNA of *S. jamaicensis* was amplified by PCR technique using a thermal cycler (Perkin Elmer, Model 9600). Ten oligonucleotide primers were designed by using Oligo Analysis Tool (http://www.operon.com/tools/oligo-analysis-tool.aspx) as shown in Table 1. A 20 µl total volume of the PCR mixture consisted of 25 ng template DNA (1 µl), 10 µl of GoTaq® Green Master Mix obtained from Promega, USA containing 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3mM $MgCl_2$), 5 µM primer for 4 µl and 5 µl of sterile water. The cycling condition was performed by an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 45°C for 45 sec and a final extension 72°C for 7 min. The reaction products were separated by electrophoresis on a 1.5% agarose gel with the 100 bp DNA marker (Promega) and visualized by UV-light after ethidium bromide staining.

Table 1 Arbitrary primers for HAT-RAPD analysis

Primers	Sequences
C1	ACGGGCGCCA
C2	ACGGGCCCCA
C3	ACGGCCGCCA
C4	ACGGCCCCCA
C5	ACGCGCGCGA
C6	CCAGGCCAGG
C7	GCAGGCCACG
C8	GCAGCGCACG
C9	GCCAGCACCG
C10	GGGAGCAGGG

Data analysis

The DNA patterns of HAT-RAPD were scored as present (1) or absent (0). The data obtained was used for haplotypes, % polymorphic loci, expected heterozygozity and AMOVA analysis implemented in GenAlEx version 6.5 [14]. Phylogenetic tree was analyzed by a Neighbor-Joining method using MEGA version 6.06 [17].

Results

The plant of *S. jamaicensis* were collected from four regions of Thailand and basically identified by external morphology according to the method of Ingole (2011) [9]. It showed dichotomously branching, lower branches woody and young branches hairy. Their leaves showed coarse teeth along the edges, and their flowers were bluish-violet (Figure 1B).

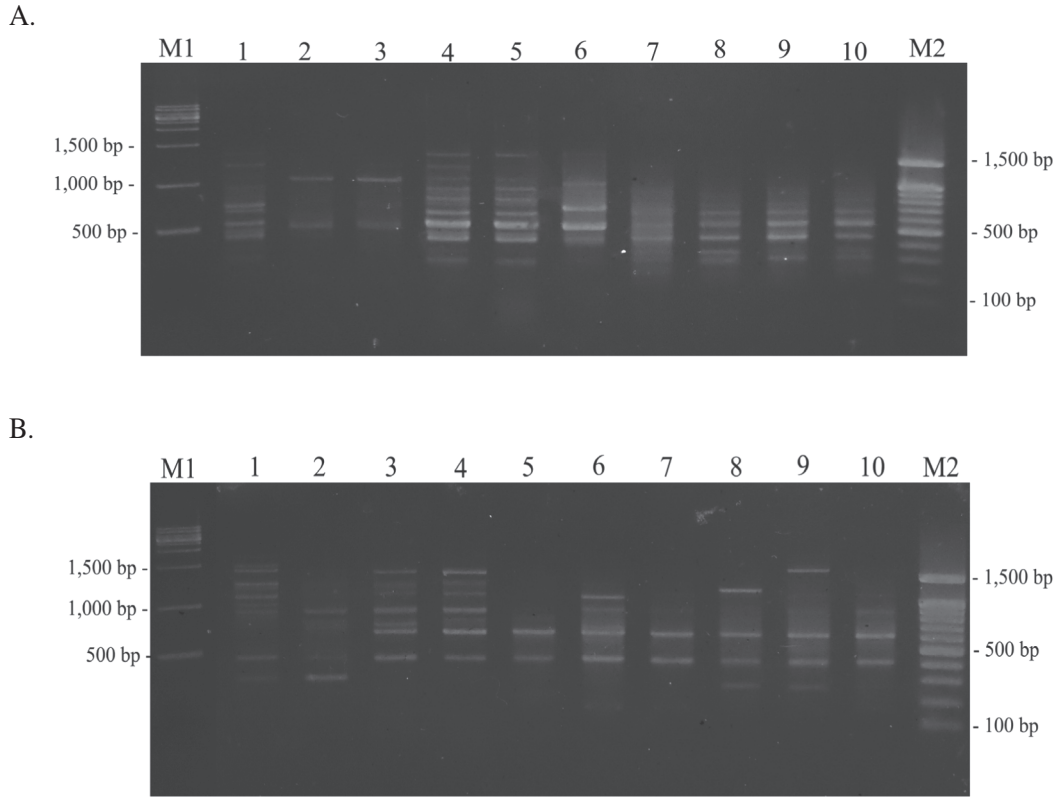

Figure 2. Examples of HAT-RAPD patterns amplified by C9 (A) and C10 (B) primers of *S. jamaicensis.* from four geographic regions in Thailand; Nakorn Nayok (lanes 1-3, A and 1-2B), Pathum Thani (lanes 4-5, A and 3-4, B), Nong Khai (lanes 6B) and Srakaew (lanes 7-10, A and 5-10, B).

For the HAT-RAPD technique, ten arbitrary primers were screened to determine genetic patterns of *S. jamaicensis.* Among them, the C9 and C10 primers produced 9 and 8 clear polymorphic HAT-RAPD markers of 300-1,500 bp length and amplified all DNA samples of S. jamaicensis for 100% (39/39). Genetic data obtained from each primer was then analyzed together. The polymorphic HAT-RAPD markers of each *S. jamaicensis* were clearly investigated as shown Figure 2A and 2B. From Table 2, one private band of the HAT-RAPD patterns was found in all tested individuals of *S. jamaicensis.* The number of the HAT-RAPD bands were found in each population varied from 10 to 13 bands. The percentages of polymorphic bands and expected heterozygosity were the greatest in Nakorn Nayok populations (P = 64.71% and H_e = 0.224), while, other populations revealed low level of the genetic diversity (P = 11.76% and H_e = 0.048 for Pathum Thani, P = 29.41% and H_e = 0.080 for Srakaew, P = 17.65%, and H_e = 0.066 for Nong Khai). In addition, nineteen of the HAT-RAPD patterns were found in *S. jamaicensis* tested.

Table 2. Genetic diversity in four populations of *S. jamaicensis* in Thailand based on polymor phisms of the HAT-RAPD method by using C9 and C10 primers

Populations	Sample size	No. of bands	No. of polymerphic bands	Percentage of polymorphic bands (%)	Mean H_e (SE)
Nakorn Nayok	12	13	11	64.71	0.224 (0.045)
Pathum Thani	9	11	10	11.76	0.048 (0.033)
Srakaew	13	11	11	29.41	0.080 (0.035)
Nong Khai	5	10	9	17.65	0.066 (0.038)

The Nei's genetic distance among pairs of populations varied from 0.106 (between Srakaew and Nong Khai) to 0.474 (between Pathum Thani and Srakaew). Neighbor-joining trees were generated from polymorphic RAPD markers (Figure 3).

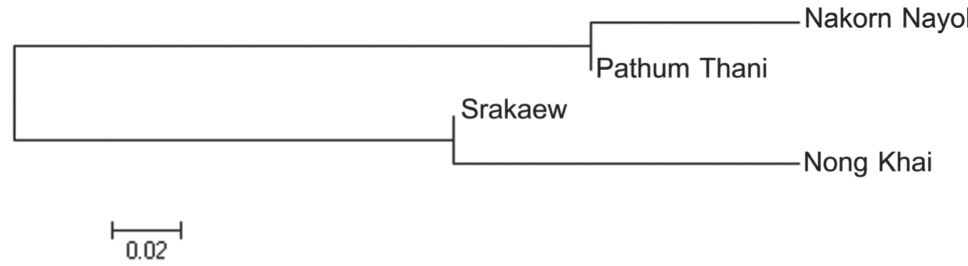

Figure 3. A neighbor-joining tree summarizing genetic relationships among four different populations of *S. jamaicensis* in Thailand from the HAT-RAPD method amplified by C9 and C10 primers.

The Analysis of Molecular Variance (AMOVA) according to the two genetic clusters of *S. jamaicensis* suggested that the high level of significant genetic differentiation was found among individuals within populations (ϕ_{PT} = 0.619, P = 0.001) and among populations (ϕ_{PR} = 0.493, P = 0.001), while low genetic variations between the two clusters (ϕ_{RT}) was obtained for 0.248 (P = 0.001) (Table 3). In case, we treated four populations (Nakorn Nayok, Pathum Thani, Srakaew and Nong Khai) as one group; it also provided high genetic variation among individuals (ϕ_{PT} = 0.587, P = 0.001).

Table 3. AMOVA analysis of *S. jamaicensis* from 4 locations of Thailand based on polymorphisms of the HAT-RAPD method amplified by C9 and C10 primers (999 random permutations, $N = 39$). Four geographic populations used in this study were divided into 2 hierarchical regions; A (Pathum Thani and Nakorn Nayok group) and B (Srakaew and Nong Khai group)

Source	df	SS	MS	Est. Var.	%	ϕ (*P*-value)
Among Regions	1	33.099	33.099	0.895	25%	$\phi_{RT} = 0.248$ ($P < 0.001$)
Among Pops	2	26.173	13.087	1.338	37%	$\phi_{PR} = 0.493$ ($P < 0.001$)
Within Pops	35	48.112	1.375	1.375	38%	$\phi_{PT} = 0.619$ ($P < 0.001$)

df = degrees of freedom, SS = sums of squares, MS = mean squares, Est. Var. = estimated variance within and among populations, % = percentage of observed variance within or among populations

Conclusion and Discussion

From our results, the high level of polymorphisms and 19 haplotypes were obtained by the HAT-RAPD method. It indicated that the high level of genetic diversity was found in *S. jamaicensis.* Moreover, the HAT-RAPD analysis is an effective technique for determining intra-genetic variations in each population of *S. jamaicensis.* This technique has been applied to estimate genetic diversity of many plants in Thailand. It has been reported that the HAT-RAPD method could be used to identify and characterize various plant species [6, 16]. The advantage of the HAT-RAPD provided more clearly, stable and reliable DNA bands than RAPD [6]. Moreover, phylogenetic analysis unambiguously demonstrated two clusters of *S. jamaicensis* in Thailand according to their geographic distribution, one composed of Nakorn Nayok and Pathum Thani and other composed of Srakaew and Nong Khai. The two clusters Neighbor-joining trees based on genetic distances of polymorphic HAT-RAPD patterns revealed level of genetic differentiation of *S. jamaicensis* in Thailand. The HAT-RAPD markers showed geographically consonant clusters because Nakorn Nayok and Pathum Thani, which were neighboring populations, were clustered together. Thus, it may exist of different evolutionary lineage of *S. jamaicensis* which is result of HAT-RAPD patterns found in this study. The AMOVA analysis with 999 random permutations of polymorphic HAT-RAPD markers could demonstrate significant genetic differentiations of *S. jamaicensis* between two geographic regions, among

populations and each individual within population ($P<0.001$). It indicated that the intra-specific genetic structure of *S. jamaicensis* in Thailand has existed. These may be influenced from geographic boundaries such as river and mountain to restrict species distribution of any organisms such as insects [19].

In conclusion, this study provided the new knowledge involving high intra-genetic polymorphisms and slightly genetic differences between geographic regions of *S. jamaicensis* from four populations of Thailand. This baseline may be used for management a conservative program, further work on developing DNA markers and application in folk medicine in communities.

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