MACROSCOPIC-MICROSCOPIC CHARACTERISTICS AND AFLP MARKER FOR IDENTIFICATION OF *TINOSPORA CRISPA* AND *TINOSPORA BAENZIGERI* ENDEMIC TO THAILAND

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ABSTRACT:

Background: *Tinospora crispa* Miers ex Hook. F & Thomson and *Tinospora baenzigeri* Forman were used in traditional medicine for treatment of antipyretic and various ailments. According to the similarity in their morphology and vernacular name, the identification was important for their effectiveness.

Methods: Macroscopic-microscopic characteristics and AFLP marker of *Tinospora crispa* and *Tinospora baenzigeri* were evaluated.

Results: Macroscopic determination revealed that *T. crispa* had more prominently tuberculate on stem than *T. baenzigeri.* The leaf shape of *T. crispa* was cordate but *T. baenzigeri* was cordate or reniform and showed a two node appearing at leaf base. However, both of them showed anomocytic stomata type but *T. crispa* presented only on abaxial epidermis whereas *T. baenzigeri* presented on both adaxial and abaxial epidermis. Leaf constant numbers of these two species showed individual value. A total of 476 AFLP bands ranging in size from 50 to 800 base pairs were generated from 5 AFLP primer combinations, of which 457 bands were polymorphic (96%). The primer combination of E+ACG/M+CTT produced the highest number (125 bands) of AFLP bands. The similarity index ranged from 0.472 to 0.934. The dendrogram generated from UPGMA indicated that *T. crispa* and *T. baenzigeri* were clearly separated from each other.

Conclusion: Macroscopic-microscopic characteristics and AFLP marker could be used for identification of these 2 *Tinospora* species. The information provided from this study can be used for identification of medicinal plants.

Keywords: Genus *Tinospora*; Macroscopic-microscopic characteristics; AFLP

DOI: 10.14456/jhr.2017.18 *Received: August 2016; Accepted: September 2016*

INTRODUCTION

The *Tinospora* is an important genus belong to the family Menispermaceae in the major group of Angiosperms. This genus consists of approximately 30 species and a few species are potential medicinal importance. They are generally climbing or twining shrubs distributed throughout the tropical and subtropical regions of Africa, Asia, Australia and the Pacific [1]. As a therapeutic agent, *Tinospora* species have been used by various cultures as traditional medicine for antipyretic, anti-inflammation, antimicrobial, antimalarial and antihyperglycemia properties [2, 3]. In Thailand,

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Cite this article as: Katib S, Ruangrungsi N, Palanuvej C, Chareonsap PP, Rungsihirunrat K. Macroscopic-microscopic characteristics and AFLP marker for identification of *Tinospora crispa* and *Tinospora baenzigeri* endemic to Thailand. J Health Res. 2017; 31(2): 143-9. DOI: 10.14456/jhr.2017.18

(A) **(B)**

Figure 1. *Tinospora crispa* (A) and *Tinospora baenzigeri* (B)

Tinospora crispa (L.) Miers ex Hook. F & Thomson known as Boraped and *Tinospora baenzigeri* Forman known as Ching cha chali, were widely used as folk medicine (Figure 1). These two species are closely related with their morphology. *T. crispa* was found in primary rainforests or mixed deciduous forests throughout a large part of Asia and Africa including all parts of Thailand [4]. It has been used as traditional medicine to treat fever, reducing thirst, increasing appetite, cooling down the body temperature and maintaining good health [5-6]. The species of *T. baenzigeri* was a deciduous climbing shrub indigenous to tropical Indian subcontinent and commonly found in hedges. This plant is widely used in the Ayurvedic system of medicine for its general tonic, anti-inflammatory, anti-arthritic, anti-allergic, anti-malarial, anti-diabetic, and aphrodisiac properties. The stem is commonly used for Ayurvedic preparations used in general debility, dyspepsia, fever, and urinary diseases [7].

The correct identification of herbal plant is the first step in quality control as recommended by WHO. There are many methods have been used for plant identification. Macroscopic and microscopic is one of the methods for examination of plant identity. Recently, the DNA markers become the marker of choice for the study of genetic diversity. Molecular techniques are being developed to more precisely, quickly and inexpensive for assessing the genetic variation. DNA markers had their advantages and disadvantages of their applications. However, some techniques are more appropriate than others depend on the specific approach and desirable properties. Amplified fragment length polymorphism (AFLP) technique has its strengths on high genomic abundance, reproducibility and generation of many polymorphic bands. AFLP can be applied in study involving genetic identity, identification, and phylogenetic relationship [8].

According to the similarity in morphological characteristics of these two species, it is difficult to identify of each other. Thus, using of several characteristics such as macroscopic and microscopic characteristics and molecular analysis has been introduced in this study for correct identification and quality control of these herbal plants. This study aims to examine the macroscopic-microscopic characteristics as well as AFLP fingerprint for identification of these two *Tinospora* species endemic to Thailand.

MATERIALS AND METHODS

Plant materials

Both fresh mature and fresh young leaves of 2 *Tinospora* species (*Tinospora crispa* and *Tinospora baenzigeri*) were collected and each of them were from 3 different locations in Thailand. Plant specimens were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrangsi, College of Public Health Sciences, Chulalongkorn University and Faculty of Pharmacy, Rangsit University, Thailand. Plants were comparing with the herbarium specimens at Forest Herbarium Thailand (BKF) and Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The details of plant specimens used in this study were shown in Table 1.

Table 1 List of plant specimens used in this study

Scientific name	Vernacular name	Location	Collecting date
Tinospora crispa (L.) Miers ex Hook. F & Thomson	Borraped (บอระเพ็ด)	1. Petchaburi(TCR-1)	October, 2013
		2. Bangkok (TCR-2)	May, 2013
		3. Pathumthani(TCR-3)	May, 2013
Tinospora baenzigeri Forman	Ching cha chali (ชิงช้าชาลี)	1. NakhonPathom(TBA-1)	June, 2013
		2. Pathumthani(TBA-2)	May, 2013
		3. Nonthaburi (TBA-3)	July, 2013

Table 2 The morphological characteristics of *T. crispa* and *T. baenzigeri*

Part of plant	*Tinospora crispa*	*Tinospora baenzigeri*
Stem	Climberand more prominently tuberculate	Climberand less prominently tuberculate
Leaf	Cordate, alternate, caudate apex, entire margin, cordate base and 8-10 cm long petiole	Cordate or reniform, caudate apex, alternate, entire margin, cordate base, 5-7 cm long petiole and two node appearing at leaf base
Flower	Inflorescences spike, 3 petals and greenish-yellow color	Inflorescence spike, 3 petals and greenish-yellow color
Fruit	Ellipsoidal, smooth, 1-2 cm long with dark yellow color	Ellipsoidal, smooth, 1-1.5cm long with dark yellow color
Seed	Moon seed, rough, 0.5-1 cm long with black color	Moon seed, rough, 0.5-1 cm long with black color

Sample preparation for macroscopic and microscopic determination

Macroscopic and microscopic characteristics of each specimen were evaluated. Macroscopic characteristics were visual inspected, photographed, and recorded. Microscopic characteristics were observed using microscope (Zeiss Axio Imager A2, Germany) according to the World Health Organization standard guideline [9]. The mature leaves of plant specimens were cleaned and leaf portion between midlib and margin were cut into small fragments (1x1 cm) and then immersed in 3% sodium hypochlorite solution until chlorophyll was removed. Plant fragments were cleared by gentle warming with chloral hydrate solution (chloral hydrate: distilled water, 8:2) until they transparent. The cleared fragments were mounted in 50% glycerine on glass slide and then examined under microscope with an attached digital camera (Cannon Power Shot A640). Leaf constant numbers such as stomatal number, stomatal index, palisade ratio, epidermal cell number and epidermal cell area were examined according to the method describe by Evans [10] with some modifications. Thirty fields of each individual specimen were determined.

The number of stomata per square millimeter (mm^2) was counted in 30 fields for individual specimen. Stomatal index was obtained by the percentage of stomata from the total number of epidermal cells. Palisade cells lying beneath 4 contiguous epidermal cells were counted and then divided by 4 to obtain the palisade ratio. Epidemal cell number and epidermal cell area per square millimeter was counted for each 30 fields. Leaf constants were calculated and expressed as mean ± standard deviation (SD) for each parameter.

DNA extraction and AFLP analysis

Fresh young leaves of each sample (50-100 mg) were individually ground in liquid nitrogen with mortar and pestle to obtain a fine powder for DNA extraction. Total genomic DNA was isolated using a modified CTAB protocol [11]. DNA quantification was performed using a Hoefer DyNA Quant 200 spectrophotometer (Pharmacia Biotech, Piscataway, NJ, USA).

The AFLP procedure was carried out according to the method described by Vos et al. [12] with a few modifications. Briefly, approximately 100 ng/µl of DNA was digested by two restriction enzymes; *Eco*RI (Roche, 10 U/µl) and *Tru*9I (Roche, 10 U/µl) in 10X buffer A (Promega) and incubated for 1 h at 37ºC. The restricted DNA fragments were ligated (with T4 DNA-ligase) to *Eco*RI (5 pmol/µl) and *Mse*I (50 pmol/µl) adapters in a final volume of 50 µl. Ligation was performed at least 3 h at 37ºC to generate DNA template for amplification. The resulting mixture (5 µl of 1:10 diluted) was used as template in a pre-amplification reaction using *Eco*RI+A and *Mse*I+C primers. The pre-amplified

Table 3 Leaf constant numbers of *T. crispa* and *T. baenzigeri*

Tinospora species	Type of stomata		Stomatal number Mean±SD (Min-Max)		Stomatal index Mean±SD (Min-Max)		Palisade ratio Mean±SD (Min-Max)	Epidermal number Mean±SD (Min-Max)	Epidermal cell area Mean±SD (Min-Max)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
T. crispa	Absent	Anomocytic	Absent	190.40±21.65 (156-236)	Absent	12.13±0.97 (10.60-13.85)	8.30±0.46 (7.50-9.00)	747.20±34.46 (668-796)	1,341.18±64.02 (1,256.28-1,497.00)
T. baenzigeri	Anomocytic	Anomocytic	16.93±6.27 (8-28)	84.66±9.80 (64-100)	2.09±0.74 (0.9-3.48)	10.41±0.80 (8.55-12.06)	6.40±0.62 (5.25-7.50)	798.06±34.39 (724-868)	1,243.11±55.46 (1,116.07-1,351.35)

Table 4 Primer combination, the number of AFLP bands, size range and the percentage of polymorphic bands

Primer combination	No. of AFLP band	Size range(bps)	No. of polymorphic bands	Polymorphism (%)
E+ACG/M+CTT	125	50 - 800	123	98.40
E+ACG/M+CTG	82	50 - 800	79	96.34
E+ACG/M+CAA	75	50 - 800	68	90.67
E+ACG/M+CGC	87	50 - 800	85	97.70
E+ACC/M+CTA	107	50 - 800	102	95.33
Total	476	50 - 800	457	96.01

Table 5 Jaccard's coefficient similarity index between *T. crispa* and *T. baenzigeri* based on AFLP markers

Species		(1) TCR-1	(2) TCR-2	(3) TCR-3	(4) TBA-1	(5) TBA-2	(6) TBA-3	(7) SNV
TCR-1	(1)	1.000						
TCR-2	(2)	0.816	1.000					
TCR-3	(3)	0.915	0.820	1.000				
TBA-1	(4)	0.484	0.472	0.480	1.000			
TBA-2	(5)	0.481	0.476	0.483	0.925	1.000		
TBA-3	(6)	0.486	0.485	0.488	0.920	0.934	1.000	
SNV	(7)	0.143	0.159	0.144	0.160	0.159	0.158	1.000

TCR= *T. crispa*, TBA= *T. baenzieri* and SVN= *Strychnos nux-vomica*

product (3 µl of 1:9 diluted) was used to selective amplification mixtures in a final volume of 10 µl. The step of amplification reactions were performed on a Px2 Thermal Cycler (Thermo Electron Coporation, USA). Amplification products of combination primers were size-fractionated on a 6% denaturing polyacrylamide gel electrophoresis using a Sequi-Gen GT Sequencing Cell (Bio-Rad, USA). The AFLP fragments were visualized with silver staining [13] and fingerprints were analyzed.

AFLP data analysis

AFLP bands were visually scored as a binary character for its present (1) or absence (0). A genetic similarity matrix was computed according from Jaccard's coefficient [14] for all pair-wise comparison in each species. A dendrogram was constructed from the matrix of similarity coefficients using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) by FreeTree software [15]. For resampling data, the values on the branches were the result of 1,000 bootstrap replication.

RESULTS AND DISCUSSION

Macroscopic and microscopic characteristics

Herbal medicine plays an important role in primary health care system. The correct identification of herbal plant is the first step in quality control as recommended by WHO. Macroscopic and microscopic is one of the methods for examination of plant identity.

The morphological characteristics of *T. crispa* and *T. baenzigeri* were summarized in Table 2. The flower, fruit and seed of *T. crispa* and *T. baenzigeri* were similar but stem and leaf were slightly difference. The stem of *T. crispa* was climber and more prominently tuberculate whereas *T. baenzigeri* had less prominently tuberculate. The leaf shape of *T. crispa* was cordate but *T. baenzigeri* was cordate or reniform and showed a two node appearing at leaf base as previously reported in *Tiliacora* and *Tinospora* in family Menispermaceae [1].

Leaf constant numbers including stomata number, stomatal index, palisade ratio, epidermal cell number and epidermal cell area of *T. crispa* and *T. baenzigeri* were evaluated under microscope and presented in Table 3.

Both *T. crispa* and *T. baenzigeri* showed anomocytic stomata type which corroborated with the characteristic of family Menispermaceae [16], but *T. crispa* presented only on lower (abaxial) epidermis whereas *T. baenzigeri* presented on both lower and upper (adaxial) epidermis. Leaf constant numbers of these two species showed individual value. The stomatal number on lower epidermis in *T. crispa* is 190.40±21.65 higher than the stomatal number in *T. baenzigeri* on both lower (84.66±9.80) and upper (16.93±6.27) epidermis. *T. crispa* showed the higher stomatal index (12.13±0.97) than *T. baenzigeri* (10.41±0.80) on the lower epidermis. Moreover, *T. crispa* had the polygonal cells shape of epidermis on both upper and lower surface, while *T. baenzigeri* had considerably different cells shape of epidermis on upper surface (polygonal cell shape) and lower surface (irregular cell shape). This characteristic can be used for identification of *T. crispa* and *T. baenzigeri.* The palisade ratio is one of the leaf measurement parameter used for identification. The palisade ratio of *T. crispa* (8.30±0.46) was higher than *T. baenzigeri* (6.40±0.62). The epidermal cell number of *T. crispa* (747.20±34.46) was less than *T. baenzigeri* (798.06±34.39) but the epidermal cell area of *T. crispa* (1,341.18±64.02) was higher than *T. baenzigeri* (1,243.11±55.46). The determination of leaf constant numbers in *T. crispa* and *T. baenzigeri* has never been previously reported. As a result, determination of leaf constant numbers was considered as one of the useful parameters to distinguish in species level. As leaf constant numbers of *T. crispa* and *T. baenzigeri* have not been previously reported, this recent study is the first report of these two species. However, leaf constant numbers have been widely used for Thai medicinal plants identification [17-19].

AFLP analysis

For AFLP analysis, a total of forty eight AFLP primer combinations were screened. Five primer combinations; E+ACG/M+CTT, E+ACG/M+CTG, E+ACG/M+CAA, E+ACG/M+CGC and E+ACC/M+CTA that could be accurately scored and highly polymorphic fragment were collected. The totals of 476 bands ranging in size from 50-800 base pairs were generated from these 5 primer combinations, of which 457 bands were polymorphic (96%). The AFLP banding was ranging from 75 to 125 bands with an average of 95.2 bands by each primer combination. The highest number of AFLP bands (125 bands) was generated from E+ACG/M+CTT and the lowest (75 bands) from E+ACG/M+CAA primer combination (Table 4).

According to the dendrogram, the genetic diversity estimates (GDEs) were used for UPGMA

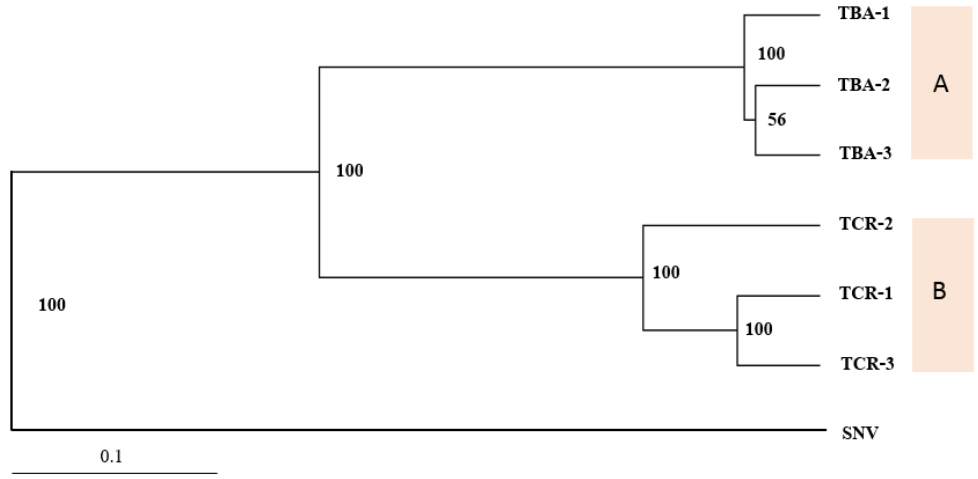

Figure 2 UPGMA dendrogram based on Jaccard's similarity coefficient of *Tinospora* species and outgroup plant. The scale bar indicated the genetic distance of 0.1

clustering by calculated from the overall Jaccard's similarity matrix obtained from five primer combinations based on AFLP analysis (Table 5). Jaccard's coefficient similarity index ranges from 0.472 to 0.934. Both *T. crispa* and *T. baenzieri* collected from 3 different locations was clearly separated from each other. *Strychnos nux-vomica* was used as outgroup plant was clearly separated from 2 *Tinospora* species (Figure 2).

AFLP marker involves the restriction of genomic DNA and followed by ligation of adaptors complementary to the restriction sites and then selective PCR amplification of the adapted restriction fragments. Polymorphisms are detected from differences in the length of the amplified fragments by polyacrylamide gel electrophoresis (PAGE) [20] or by capillary electrophoresis. AFLP analysis was very effective in detecting genetic variation in medicinal plants as well as *Tinospora* and revealed a large number polymorphic DNA fragments (96%) and the similarity index ranges from 0.472 to 0.934 in this study which similar to the result previously reported in the genetic diversity in *Tinospora cordifolia* revealed the similarity indices ranged from 0.68-1.0 for ISSR and 0.52-0.96 for EST-SSR [21]. Due to its advantages in the ability to work without sequence information, high reproducibility, multilocus detection and small amount of DNA needed makes, AFLP technique more valuable marker for the detection of genetic polymorphism and identification. AFLP markers have been successfully been used for discriminating between closely related species and authentication of some other medicinal plants such as *Zingiber* species [22]. DNA markers are more reliable because the genetic information is unique for each species and is independent of age, physiological condition and environment factors.

It can be concluded that macroscopic-microscopic and AFLP marker may be a more useful tool for the identification of *T. crispa* and *T. baenzieri* and the correlation was found between the molecular and morphological data. The present findings can help in the genetic variation analysis among closely related species.

ACKNOWLEDGEMENTS

This study was scholarly supported by the scholarships for the Ph.D. programme, Mae Fah Luang University and The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). The authors would like to thank the College of Public Health Sciences and Plant Genetic Conservation Project under The Royal Initiation of Her Royal Highness Princess Maha Chackri Sirindhorn (RSPG), Chitralada Palace, Dusit, Bangkok, 10303, Thailand for facilities support.

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