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Evaluation of Potential DNA Barcodes for Identifying *Thunbergia* spp.

Piyaporn Wongakson[1], Suang Rungpragayphan[1], Busaba Powthongchin[1]*

Abstract

Introduction: A number of *Thunbergia* species are widely used as medicinal plants. Identification by traditional morphological methods is difficult especially when specimens are commercial herbal products, which morphology is unattainable. To facilitate the identification process, this study searched for potential DNA barcodes by evaluating three DNA loci, the chloroplast *psb*A-*trn*H and *trn*L-*trn*F intergenic spacers and the second nuclear internal transcribed spacer (ITS2), among eight species of *Thunbergia*. **Methods:** Plant samples were collected and fresh leaves were used for genomic DNA extractions. Polymerase chain reaction amplifications of the *psb*A-*trn*H, *trn*L-*trn*F and ITS2 regions were performed. The PCR products were sequenced and verified by BioEdit v7.0.8 and Clustal W programs. Kimura 2-Parameter (K2P) were computed via MEGA 6.06 to reveal the genetic divergence. **Results:** Three candidate regions of all *Thunbergia* DNA samples were successfully amplified with the rate of 100 %. These amplified products were also successfully sequenced (100%) in both directions. The average interspecific divergence values calculated from K2P for *psb*A-*trn*H, *trn*L-*trn*F, and ITS2 were 0.088, 0.054 and 0.121, respectively, implying that each *Thunbergia spp*. could be discriminated from one another. **Conclusion:** This study confirmed the universality of the primers used for *psb*A-*trn*H, *trn*L-*trn*F and ITS2 PCR amplification and sequencing. The K2P results showed that each locus could individually differentiate between *Thunbergia* spp. under studied while the most variable region that can make a distinction effectively was ITS2. The combination of ITS2 with *psb*A-*trn*H and/or *trn*L-*trn*F sequences was proposed as a potential barcode for identifying *Thunbergia* species.

Keywords: *Thunbergia spp., DNA barcode, psb*A-*trn*H*, trn*L-*trn*F*, ITS2*

[1] Faculty of Pharmacy, Silpakorn University, Nakornpathom, Thailand 73000

* **Corresponding author**: Busaba Powthongchin, Faculty of Pharmacy, Silpakorn University, Nakornpathom, Thailand 73000

E-mail: powthongchin_b@su.ac.th

1. Introduction

The genus *Thunbergia*, belonging to the family *Acanthaceae*, comprises more than hundred flowering plant species (Jiaqi, et al., 2011). Only eleven species, *T. affinis*, *T. alata, T. coccinea, T. colpifera, T. erecta, T. fragrans, T. grandiflora, T. hossei, T. laurifolia*, *T. nivea* and *T. similis*, are found in Thailand (The Forest Herbarium, 2001). Most species are widely known as medicinal plants such as *T. alata, T. fragrans, T. grandiflora* and *T. laurifolia* (Meurer-Grimes, et al., 1996; Kanchanapoom, et al., 2002; Leonti, et al., 2002; Okello and Ssegawa, 2007) while some of them are ornamental plants (The Forest Herba rium-BKF, 2014). The popular one in Thai traditional medicine is *T. laurifolia* or Rang chuet (Thai vernacular name), which is commonly used as detoxifying agent (Chan et al., 2011; Suwanchaikasem, et al., 2013). Based on morphological and physiological criteria, most species of *Thunbergia* are efficiently identified particularly for fresh or well-preserved specimens. Other forms of commercial herbal products (*eg*. tea, ground or powder, etc.) cannot be correctly identified by those criteria. Therefore, some limitations in traditional morphology identification are unable to differentiate such herbs when an incomplete specimen was obtained. Thus, a novel method for simple and accurate species identification is required. Recently, a DNA-based identification system known as DNA barcode has been proposed and competently exploited to identify species and can resolve many taxonomic and evolutionary problems (Hebert, et al., 2003; Kress, et al., 2005; Miller, 2007; Kress and Erickson, 2007). It was accepted that the ideal DNA barcode should have three important characteristics as follows i) short sequence length to facilitate DNA extraction and PCR amplification, ii) flanked by conserved regions for capturing with the universal primers, and iii) contain sufficient sequence variation to discriminate between species (Kress, et al., 2005; Weising, et al., 2005). Mitochondrial cytochrome c oxidase subunit 1 (mtCO1) gene has been successfully used as universal DNA barcode to identify animal biological diversity such as birds (Hebert, et al., 2004), fish (Ward, et al., 2005), arthropods (Hajibabaei, et al., 2006) and mammals (Hajibabaei, et al., 2007). However, the mtCO1 gene did not work well in plant because of its slow evolution rate. Several plant DNA regions with high sequence variation and evolutionary rate have been proposed for identifying species such as *mat*K (CBOL Plant Working Group, 2009), *trn*L-*trn*F intergenic spacer (McDade and Moody, 1999; McDade, et al., 2005), *psb*A-*trn*H intergenic spacer (Pang, et al., 2012) and the nuclear ribosomal internal transcribed spacer 2 (nrITS2) (Gao, et al., 2010). Additionally, the combination of more than one DNA barcode is suggested for better species distinguishing such as a combination of *mat*K and *rbc*L has been recommended as the core barcode for land plants (CBOL Plant Working Group, 2009). Until now, no universal DNA barcode has been proposed for plants.

In the present study, the capability of three DNA loci: the chloroplast *psbA-trnH* and *trn*L-*trn*F intergenic spacers and ITS2 as candidate DNA barcodes for identifying *Thunbergia* spp.

were studied. The results suggested that ITS2 or combinations of ITS2 with *psbA-trnH* and/or *trn*L-*trn*F *can effectively discriminate among each Thunbergia spp.*

2. Materials and Methods

Plant materials

The plant samples (1 sample for *Thunbergia alata*, *T. coccinea, T. colpifera, T. fragrans* and *T. hossei*. and 3 samples for *T. erecta, T. grandiflora* and *T. laurifolia*) were collected from various areas of Thailand as shown in Table 1. Morphological identification of these samples followed the Flora of China guideline (Jiaqi, et al., 2011) and the Encyclopedia of plant in Thailand database (The Forest Herbarium-BKF, 2014). Herbarium specimens were well prepared according to a standard protocol (Chayamarit, 2001). All corresponding voucher images and specimens were kept at Silpakorn University and subse quently the herbaria will be deposited at the Forest Herbarium-BKF, Thailand.

Table 1: Plant materials used in this study

Scientific name	Thai vernacular name	Location in Thailand	Voucher ID
Thunbergia alata	Waeo ta	Chiang Rai	PW058
T. coccinea	Nam nae daeng	Chiang Rai	PW062
T. colpifera	Rang chuet ton phuka	Nan	PW070
T. erecta	Chong nang	Bangkok, Nakhon Pathom	PW071, PW089, PW090
T. fragrans	Nam nae khao	Chiang Mai	PW073
T. grandiflora	Soi in thanin	Bangkok, Nakhon Pathom	PW072, PW091, PW092
T. hossei	Nam nae dong	Chiang Mai	PW049
T. laurifolia	Rang chuet	Ayutthaya, Chiang Rai, Nakhon Pathom	PW037, PW093, PW094

DNA extraction and PCR amplification

Total DNA was extracted, using Genomic DNA mini kit plant (Geneaid Biotech Ltd., Taiwan), from 300 mg of fresh leaves, which was pulverized under liquid nitrogen. Quality and rough quantity of extracted DNA were determined by electrophoresis on 0.8% agarose gel stained with ethidium bromide.

Polymerase chain reaction (PCR) amplifications of the *psb*A-*trn*H, *trn*L-*trn*F and ITS2 regions were performed on a Gene-Amp PCR System 9700 (Applied Biosystems, USA) using the primer pairs and thermocycling conditions obtained from previous studies given in Table 2. A 50 µl reaction mixture containing 10-100 ng DNA template, 1 µM of each primer, 1x PCR

buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 2.5 unit of *Taq* DNA polymerase (Invitrogen, USA). The PCR products were confirmed by 2% agarose gel electrophoresis and then purified by using Geneaid Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan).

Table 2: Primers and reaction conditions

DNA region	Primer pairs	Primer sequence (5'-3')	Thermocycling conditions
*psb*A-*trn*H	*psb*A'3f	GTTATGCATGAACGTAAT-GCTC	94°C 5 min; [40 cycles: 94°C 40 s; 55°C 40 s; 72°C 40 s]; 72°C 10 min
(Chen, et al., 2010)	*trn*H'r	C G C G C A T G G T G G A T-TCACAATCC	
*trn*L-*trn*F	UniE	GGTTCAAGTCCCTCTATCCC	94°C 5 min; [30 cycles: 94°C 30 s; 50°C 40 s; 72°C 40 s]; 72°C 10 min
(McDade and Moody, 1999)	UniF	ATTTGAACTGGTGACACGAG	
ITS2	S2F	ATGCGATACTTGGTGTGAAT	94°C 5 min; [40 cycles: 94°C 30 s; 56°C 40 s; 72°C 40 s]; 72°C 10 min
(Chen, et al., 2010)	S3R	GACGCTTCTCCAGACTACAAT	

DNA sequencing and analysis

DNA sequencing in both directions using the same primers for PCR amplification were performed on the DNA Sequencer at SolGent Co., Ltd. (Korea). The forward and reverse sequences obtained were edited and aligned using the program Bioedit v7.0.8 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html; Hall, 1999) and Clustal W (Larkin, et al., 2007), respectively. The genetic distances were computed using MEGA 6.06 according to the Kimura 2-Parameter (K2P) model (Kimura, 1980; Tamura, et al., 2013).

3. Results and Discussion

Specimen collection and morphological verification of Thunbergia spp.

Although it was reported that there are eleven species of *Thunbergia* existing in Thailand (The Forest Herbarium, 2001), in this study only eight species, *T. alata*, *T. coccinea*, *T. colpifera*, *T. erecta*, *T. fragrans*, *T. grandiflora*, *T. hossei* and *T. laurifolia* could be collected from various areas in Thailand (see Fig. 1 and Table 1). *T. affinis, T. nivea*, and *T. similis* could not be found even with great effort. Morphological identification of the plant specimens conformed to the Flora of China guideline (Jiaqi, et al., 2011) and the Encyclopedia of plant in Thailand database (The Forest Herbarium-BKF, 2014). Stalks, leaves, flowers, and fruits of each specimen were used

to prepare the herbarium. All corresponding voucher images and specimens were kept at Silpakorn University and subsequently the herbaria will be deposited at the Forest Herbarium -BKF, Thailand.

By means of morphology examination of fresh specimen, each species of *Thunbergia* was easily identified except for *T. grandiflora* and *T. laurifolia* because of the similarity of their flowers, as shown in Fig. 1. So, other features especially the shape of leaves could be used to differentiate between them. *T. laurifolia* has an oval-shaped leaves while *T. grandiflora* leaves are variable in shape, *i.e*. heart or triangular-shaped with broad-base and narrow to a pointed tip, the margins may be toothed, lobed or entire (The Forest Herbarium-BKF, 2014). Therefore, it must be noted that to verify any plant, a complete specimen should be obtained otherwise it might lead to misunderstanding or eventually misuse, especially for the plants appeared to be very similar. This problem emphasizes an important aspect for verification or quality control of commercial herbal products, either in the form of fresh or processed herb. A new molecular technique known as 'DNA barcoding' has been proposed as a simple and accurate technique for identifying the species of living organisms (Hebert, et al., 2003). Herein, we applied this method to identify *Thunbergia* spp. by investigating three DNA loci as potential DNA barcodes.

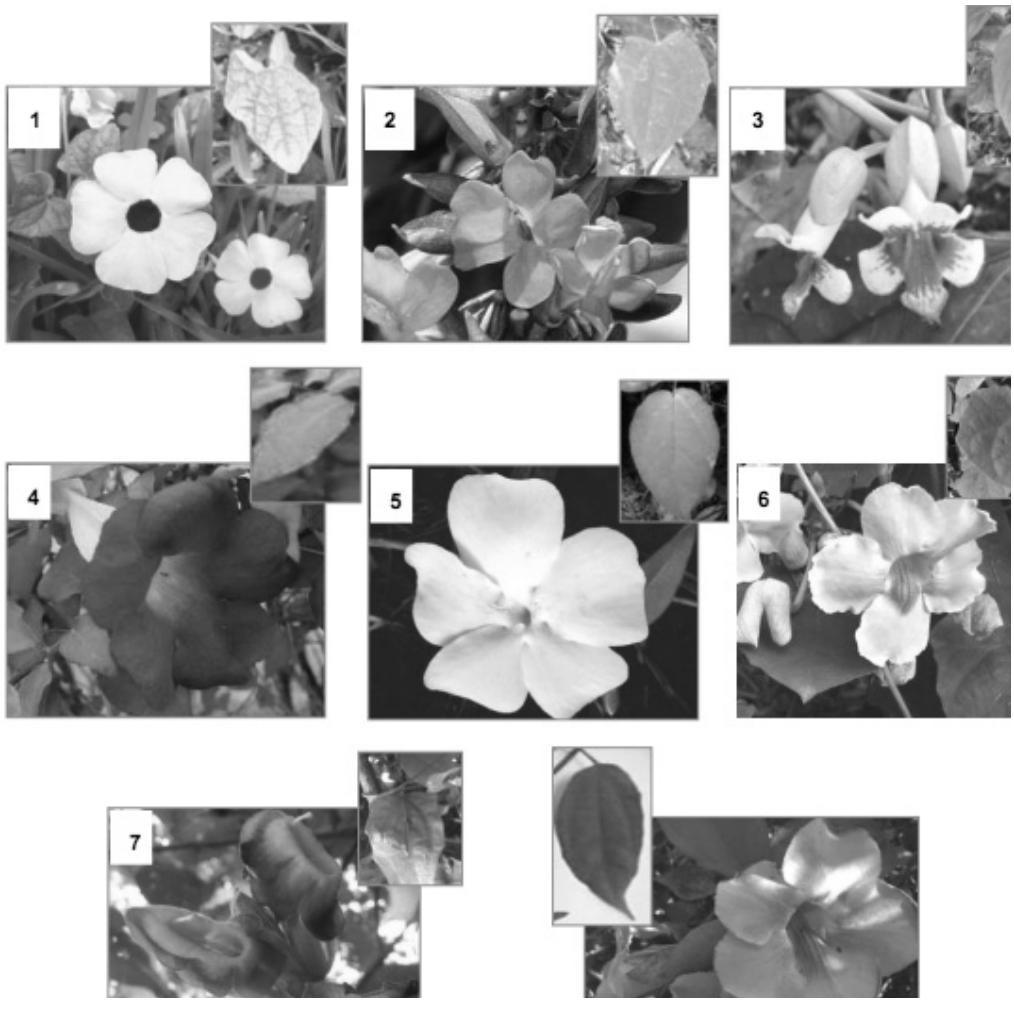

Figure 1: The images of *Thunbergia* spp. used in this study showing their flowers and leaves: 1) *T. alata* 2) *T. coccinea* 3) *T. colpifera* 4) *T. erecta* 5) *T. fragrans* 6) *T. grandiflora* 7) *T. hossei* and 8) *T. laurifolia*

DNA extraction and PCR amplification

Fresh leaves were used for DNA extraction because of their availability and ease of extraction. Purified genomic DNA was then used as a template for PCR amplifications. The primer pairs and reaction conditions from previous studies (McDade and Moody, 1999; Chen, et al., 2010) were successfully amplified *psb*A–*trn*H, *trn*L-*trn*F and ITS2 regions of these *Thunbergia* DNA samples with 100% success rate. As estimated from agarose gel electrophoresis, the sizes of PCR products of *psb*A-*trn*H, *trn*L-*trn*F, and ITS2 were 550, 450 and 500 bps, respectively (see Fig.2). High success rate of PCR amplification supported the 'universality' of these primers to be used as the universal primers for amplifying these regions.

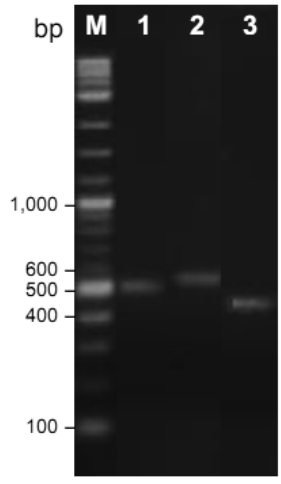

Figure 2: PCR products of ITS2, *psb*A-*trn*H, and *trn*L-*trn*F amplification. The figure show 2 % agarose gel electrophoresis (ethidium bromide-stained) of 500 bp, 550 bp and 450 bp fragments of ITS2 (lane 1), *psb*A-*trn*H (lane 2), and *trn*L-*trn*F (lane 3) amplification, respectively. Lane M represents 2-Log DNA ladder.

Sequencing efficiency and sequence analysis

All purified PCR products obtained could be sequenced in both directions giving overall success sequence rate of 100%. The accuracy of target sequences were verified by editing and alignment between the forward and reverse sequences obtained using the programs BioEdit v7.0.8 and Clustal W, respectively and then were carefully fine-tuned manually. These results revealed the sequence quality of these PCR products. The lengths of the intergenic spacers *psb*A-*trn*H, *trn*L-*trn*F and the second nuclear ribosomal internal transcribed spacer, ITS2 sequences ranged from 423 to 448 bps (avg. = 440 bps), 319 to 349 bps (avg. = 338 bps) and 207 to 226 bps (avg. = 219 bps), respectively (Table 3). It was noted that the length of these three DNA regions were quite short enough to be easily amplified and sequenced, thus appropriate for DNA barcoding in the study of *Thunbergia* spp.

Table 3: The length (base-pairs) and % GC content of psbA-trnH, trnL-trnF, and ITS2 from Thunbergia spp.

	length (base-pairs)			%GC content		
	***psb*A-*trn*H**	***trn*L-*trn*F**	**ITS2**	***psb*A-*trn*H**	***trn*L-*trn*F**	**ITS2**
Thunbergia alata	446	337	223	26.90	37.98	70.40
T. coccinea	440	345	218	25.68	37.68	70.64
T. colpifera	423	319	217	26.24	39.49	69.12
T. erecta	441	340	207	27.21	37.64	69.56
T. fragrans	446	349	226	27.88	39.95	72.57
T. grandiflora	440	335	222	25.90	37.91	63.58
T. hossei	448	340	217	27.00	37.64	73.27
T. laurifolia	432	341	225	26.85	37.24	71.11
Average	**440**	**338**	**219**	**26.71**	**38.19**	**70.03**

Another important feature of a desirable DNA barcode is an ease to be amplified, hence its GC content should be concerned. If a DNA fragment has too high GC content, it is rather difficult to amplify using a standard PCR protocol, and usually need optimization. By analyzing percent GC content of three DNA loci of eight *Thunbergia* species, it was found that *psb*A-*trn*H, *trn*L-*trn*F and ITS2 sequences contained an average of 26.71, 38.19 and 70.03 %GC, respectively (Table 3). Albeit ITS2 seemed to have high GC content, it was amplified successfully. That was probably due to its relatively short sequence and primer specificity with high denaturing temperature.

It is also noted that the discriminating power among species is a significant characteristics of an ideal DNA barcode. In this work, we utilized the K2P distance (divergence, d-value) *via* the program MEGA 6.06 to demonstrate the difference between two compared sequences. The K2P distance varies between 0-1 (0<K2P distance<1). The higher the K2P value, the more the divergence (*i.e.* high sequence variation between species so that they can be discriminated from one another). All K2P values of pairwised sequences comparison between *psb*A-*trn*H, *trn*L-*trn*F, and ITS2 of eight *Thunbergia* species were shown in Table 4 with an average of 0.088, 0.054, and 0.121, respectively. The results showed that all three candidate barcodes could obviously differentiate among pairs of studied species as shown by 'K2P divergence value > 0'. In this regard, ITS2 showed the highest average K2P value implying that it was the best performing single locus amongst three candidate barcodes to identify the variation. However, for some *Thunbergia* pair *eg*. *T. alata vs.T. coccinea*

or *T. alata vs. T. hossei*, the *trn*L-*trn*F locus was found relatively better than ITS2 in differentiating these species due to its higher K2P value. The result presented here was in accordance with the previous study that proposed ITS2 as a universal DNA barcode for identification of medicinal plants. They showed that the ITS2 sequence readily differentiated all 24 medicinal species and 66 related species at the genus level and could also differentiate 80% of the studied species at the species level. Moreover, the success rate of identifying over 4800 plant species from 750 genera using the ITS2 region reached 92.7% (Chen, et al., 2010).

Table 4 Value of sequence divergences (Kimura 2-Parameter) computed between each pair of Thunbergia spp.

		*psb*A-*trn*H	*trn*L-*trn*F	ITS2
T. alata	*T. coccinea*	0.078	0.147	0.089
T. alata	*T. colpifera*	0.153	0.135	0.184
T. alata	*T. erecta*	0.068	0.139	0.146
T. alata	*T. fragrans*	0.040	0.144	0.084
T. alata	*T. grandiflora*	0.140	0.147	0.102
T. alata	*T. hossei*	0.068	0.147	0.095
T. alata	*T. laurifolia*	0.087	0.152	0.094
T. coccinea	*T. colpifera*	0.143	0.042	0.178
T. coccinea	*T. erecta*	0.099	0.024	0.127
T. coccinea	*T. fragrans*	0.086	0.031	0.126
T. coccinea	*T. grandiflora*	0.090	0.000	0.027
T. coccinea	*T. hossei*	0.039	0.000	0.056
T. coccinea	*T. laurifolia*	0.016	0.003	0.010
T. colpifera	*T. erecta*	0.095	0.024	0.206
T. colpifera	*T. fragrans*	0.141	0.046	0.193
T. colpifera	*T. grandiflora*	0.074	0.042	0.159
T. colpifera	*T. hossei*	0.173	0.042	0.184
T. colpifera	*T. laurifolia*	0.150	0.046	0.165
T. erecta	*T. fragrans*	0.066	0.028	0.199
T. erecta	*T. grandiflora*	0.090	0.024	0.146
T. erecta	*T. hossei*	0.089	0.024	0.133
T. erecta	*T. laurifolia*	0.108	0.028	0.133
T. fragrans	*T. grandiflora*	0.079	0.031	0.126
T. fragrans	*T. hossei*	0.099	0.031	0.139
T. fragrans	*T. laurifolia*	0.089	0.035	0.133
T. grandiflora	*T. hossei*	0.033	0.000	0.073
T. grandiflora	*T. laurifolia*	0.019	0.003	0.016
T. hossei	*T. laurifolia*	0.048	0.003	0.062
Average		0.088	0.054	0.121

* Italic numbers represent the highest value of sequence divergence among the three candidate barcodes

For *psb*A-*trn*H region, the average K2P value was 0.088 which was lower than that of ITS2. Several studies proposed *psb*A-*trn*H as a good DNA barcode candidate for plants due to its PCR priming sites within highly conserved sequences and high numbers of substitution in the sequence (Pang et al. 2012). The *psb*A-*trn*H was also found efficient in distinguishing various *Dendrobium* Sw. species, with relatively high interspecific variation (Yao et al., 2009). Moreover, it was also proposed that the plastid *psb*A-*trn*H intergenic spacer region would be a suitable universal barcode for land plants (Kress and Erickson, 2007). Therefore, *psb*A-*trn*H could also be considered as a DNA barcode for *Thunbergia* species with lower discrimination power than ITS2.

In case of *trn*L-*trn*F, it showed the lowest average sequence divergence among three tested markers. Moreover, it could not differentiate between *T. coccinea* and *T. grandiflora* or *T. hossei*; and *T. grandiflora* vs. *T. hossei*, with the K2P value of 0. These data indicated that the *trn*L-*trn*F locus appear to have no or little variation to discriminate between most of *Thunbergia* species. Chase, 2005 also showed that *trn*L-*trn*F region was not variable enough to be used as a DNA barcode (Chase, 2005). Nonetheless, *trn*L-*trn*F performed well over ITS2 and *psb*A-*trn*H to discriminate *T. alata* from *T. fragrans* or *T. grandiflora* or *T. hossei* or *T. laurifolia*, suggesting that the combination of loci such as ITS2 and *trn*L-*trn*F may be required if single ITS2-locus cannot differentiate among these species.

Concerning CBOL's data standards and guidelines for DNA barcode locus selection, ITS2 was then proposed as a potential barcode to indentify *Thunbergia* spp. due to its high potential to identify variation, easy amplification and sequencing. In addition, we suggest using two or three-locus combinations (*eg.* ITS2 and *psb*A-*trn*H or *trn*L-*trn*F, or using ITS2 together with *psb*A-*trn*H and *trn*L-*trn*F) that may be necessary to provide enough variation and increasing the discriminatory power.

4. Conclusion

In this study, we evaluated three DNA barcode candidates, *psb*A-*trn*H, *trn*L-*trn*F, and ITS2 for identification of *Thunbergia* spp. existing in Thailand. PCR amplification and sequencing efficiency, and sequence divergence of the three markers were investigated. All candidates showed great efficiency in PCR amplification and sequencing using universal primers. Although, these three genetic loci can individually differentiate most *Thunbergia* species under studied but their discrimination powers vary. ITS2 showed the highest sequence divergence while the lowest one was *trn*L-*trn*F. ITS2 seemed to be the best single DNA barcode locus, but a combination of ITS2 with *psb*A-*trn*H and/or *trn*L-*trn*F will greater improve discrimination power for identification of *Thunbergia* spp. However, we still further study by increasing the number of samples in order to standardize these potential DNA barcodes, which may be applied for authenticating or developing the quality control of related herbal products in the near future.

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References

BioEdit. [Internet]. 2013 [cited 2014 October 15]. Available from: http://www.mbio.ncsu.edu/BioEdit/BioEdit.html

CBOL Plant Working Group. A DNA barcode for land plants. *Proc Natl Acad Sci* 2009;106:12794-12797.

Chan EWC, Eng SY, Tan YP, Wong ZC. Phy tochemistry and pharmacological proper ties of *Thunbergia laurifolia*: A review. *PHCOG J* 2011;3(24):1-6.

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N, et al. Land plants and DNA barcodes: short-term and long-term goals. *Phil Trans R Soc B* 2005;360:1889-1895.

Chayamarit K. Key characters of plant families. 2nd ed. Bangkok: Arun Press; 2007.

Chen S, Yao H, Han J, Liu C, Song J, Shi L et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* 2010;5(1):e8613.

Gao T, Yao H, Song J, Liu C, Zhu Y, Ma X, et al. Identification of medicinal plants in the family *Fabaceae* using a potential DNA barcode ITS2. *J Ethnopharmacol* 2010;130:116-121.

Hall TA, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser*. 1999;41:95–98.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PD. DNA barcodes distinguish species of tropical Lepidoptera. *Proc Natl Acad Sci U S A* 2006;103:968–971.

Hajibabaei M, Singer GA, Clare EL, Hebert PD. Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. *BMC biol* 2007;5:24.

Hebert PD, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B* 2003;270:313-321.

Hebert PD, Stoeckle MY, Zemlak TS, Francis CM. Identification of Birds through DNA Barcodes. *PLoS Biol* 2004;2(10):e312.

Jiaqi H, Yunfei D, Daniel TF. *Thunbergia*. *Fl. China* 2011;19:377-379.

Kanchanapoom T, Kasai R, Yamasaki K. Iridoid glucosides from *Thunbergia laurifolia*. *Phytochemistry* 2002; 60(8):769-771.

Kimura M. A simple method for estimating evolu tionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;16:111-120.

Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: the coding *rbc*L

gene complements the non-coding *trn*H-*psb*A spacer region. *PLoS One* 2007;2:e508.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. *Proc Natl Acad Sci* 2005;102:8369-8374.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23:2947-2948.

Leonti M, Sticher O, Heinrich M. Medicinal plants of the Popoluca, México: organoleptic properties as indigenous selection criteria. *J Ethnopharmacol* 2002;81(3): 307-315.

McDade LA, Moody ML. Phylogenetic relation ships among *Acanthaceae*: evidence from noncoding *trn*L-*trn*F chloroplast DNA sequences. *Am J Bot* 1999;86(1): 70-80.

McDade LA, Daniel TF, Kiel CA, Vollensen K. Phylogenetic relationships among *Acanthaceae*: major lineages present contrasting patterns of molecular evolution and morphological differentiation. *Systematic Botany* 2005;30(4):834-862.

Meurer-Grimes B, McBeth DL, Hallihan B, Delph S. Antimicrobial activity in medicinal plants of the *Scrophulariaceae* and *Acanthaceae*. *Pharm Biol* 1996;34(4):243-248.

Miller SE. DNA barcoding and the renaissance of taxonomy. *Proc Natl Acad Sci U S A* 2007;104:434-439.

Okello J, Ssegawa P. Medicinal plants used by communities of Ngai Subcounty, Apac District, northern Uganda. *Afr J Ecol* 2007;45:76-83.

Pang X, Lin C, Shi L, Liu R, Liang D, Li H, et al. Utility of the *trn*H-*psb*A intergenic spacer region and its combinations as plant DNA barcodes: a meta-analysis. *PLoS One* 2012;7(11):e48833.

Suwanchaikasem P, Phadungchareon T, Sukrong S. Authentication of the Thai medicinal plants sharing the same common name 'Rang Chuet' : *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* aff. *amada* by combined techniques of TLC, PCR-RFLP finger-prints and antioxidant activities. *ScienceAsia* 2013;39:124-133.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolu tionary Genetics Analysis version 6.0. *MBE* 2013;30:2725-2729.

The Forest Herbarium. Thai plant names Tem Samitinand. 2nd rev.ed. Bangkok: Royal Forest Department; 2001.

The Forest Herbarium-BKF [Internet]. Bangkok: Department of National Parks, Wildlife and Plant Conservation; 2014 [cited 2014 January 8]. The encyclopedia of plants in Thailand. Available from: http://www.web3.dnp.go.th/botany/mindexdict.aspx

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PD. DNA barcoding Australia's fish species. *Philos Trans R Soc Lond B Biol Sci* 2005;360:1847-1857.

Weising K., Nybom H., Wolff K., Kahl G. DNA Fingerprinting in Plants. 2nd ed. Boca Raton: CRC Press Taylor& Francis Group; 2005.

Yao H, Song JY, Ma XY, Liu C, Li Y, Xu HX, et al. Identification of *Dendrobium* species by a candidate DNA barcode sequence: the chloroplast *psbA–trnH* intergenic region. *Planta Medica* 2009;75:667–669.