รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาวิธีการจัดจำแนกและตรวจสอบสมุนไพรไทยที่เป็นที่นิยม

Development of identification and authenticating technique and DNA bank for commonly used Thai medicinal plants

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บทคัดย่อ

พืชในวงศ์ Acanthaceae จัดเป็นไม้ล้มลุกพบมากในเขตร้อนและเขตกึ่งร้อน ประกอบด้วยสมาชิกกว่า 3,450 สปีชีส์ ใน กว่า 300 สกุล จากจำนวนดังกล่าว หลายสปีชีส์ได้ถูกนำมาใช้เป็นยาสมุนไพรในประเทศไทยซึ่งมักมีการจำหน่ายในรูปแบบที่แปรรูปแล้วซึ่งยากต่อการระบุชนิดด้วยวิธีทางสัณฐานวิทยา งานวิจัยชิ้นนี้จึงจัดทำขึ้นเพื่อศึกษาความเป็นไปได้ในการใช้เทคนิค Barcode DNA High Resolution Melting (Bar-HRM) ในการระบุชนิดพืชสมุนไพรไทยที่เป็นที่นิยมของคนไทย โดยเน้นพืชในวงศ์ Acanthaceae ซึ่งไม่ต้องอาศัยความชำนาญเชิงอนุกรมวิธานและรวดเร็ว แม่นยำ การทดลองแบ่งเป็น 2 ส่วนหลัก ส่วนแรก อ การวิเคราะห์หาตำแหน่งดีเอ็นเอบาร์โค้ดที่มีความเหมาะสมในการนำมาใช้ในการระบุชนิดพืชสมุนไพรทั่วไป และ ตัวอย่างเฉพาะพืชในวงศ์ Acanthaceae โดยเลือกตำแหน่งบนคลอโรพลาสตร์จีโนมมา 4 ตำแหน่ง ได้แก่ *matK rbcL rpoC trnL* และเลือกตำแหน่งบนนิวเคลียร์จีโนมมา 1 ตำแหน่ง คือ ITS2 ผลการศึกษาพบว่า จากการเปรียบเทียบลำดับนิวคลีโอไทด์ของดีเอ็นเอบาร์โค้ดที่เป็นที่นิยมใช้จากตำแหน่งบนคลอโรพลาสตร์จีโนม 4 ตำแหน่งพบว่า *rpo*C1 มีประสิทธิภาพดีที่สุดจากทั้ง 4 ตำแหน่งในขณะที่ *mat*K ได้ผลแย่ที่สุดในการระบุชนิดพืชในสมุนไพรไทย โดยคิดเป็นอัตราความสำเร็จ 58% สำหรับ *rpo*C1 และ 15% สำหรับ *mat*K และเมื่อลองใช้หลายตำแหน่งร่วมกัน (2-4 ตำแหน่ง)ในการระบุชนิด พบอัตราความสำเร็จอยู่ในช่วง 81–99% ทั้งนี้ขึ้นอยู่กับตำแหน่งที่นำมาใช้ร่วมกัน ส่วนจากการเปรียบเทียบลำดับนิวคลีโอไทด์ในส่วนของ ITS2 นั้นผลพบว่าอาจมีความเหมาะสมที่สุดสำหรับเทคนิค Bar-HRM เพื่อใช้แยกชนิดพืชในวงศ์ Acanthaceae เนื่องจากมีความหลากหลายของลำดับนิวคลีโอไทด์สูงสุด (88.12%) และมีสัดส่วนเบส GC สูงสุด (66.79%) ส่วนที่สองคือ การทดลองใช้เทคนิค Bar-HRM ที่พัฒนาขึ้นมานี้มาใช้ในการสุ่มตรวจสมุนไพรในท้องตลาด โดยเป็นผลิตภัณฑ์พืชในสมุนไพรไทยในวงศ์ Acanthaceae เช่น ฟ้าทะลายโจร ทองพันชั่ง และเหงือกปลาหมอ ซึ่งได้ผลสำเร็จเป็นที่น่าพอใจในการนำเทคนิค Bar-HRM ที่พัฒนาขึ้นมานี้มาใช้ในการสุ่มตรวจสมุนไพรในท้องตลาด จากการสุ่มตรวจผลิตภัณฑ์พืชในสมุนไพรไทยในวงศ์ Acanthaceae ที่วางขายโดยพบว่าผลิตภัณฑ์ฟ้าทะลาย (*Andrographis paniculata*) ที่นำมาตรวจ 10 ตัวอย่าง เป็นฟ้าทะลายโจรตามที่ฉลากระบุจริง แต่ในการสุ่มตรวจสมุนไพรมา 3 ชนิดที่นิยมใช้ซึ่งอยู่ในวงศ์ Acanthaceae โดยไม่ได้มีบรรจุการห่อที่วางขายอยู่ตามท้องตลาด จำนวน 15 ตัวอย่าง พบการปนเปื้อน 3 ตัวอย่าง ซึ่งแสดงให้เห็นว่าเทคนิคที่พัฒนาขึ้นมานี้สามารถนำมาประยุกต์ใช้สำหรับตรวจสอบผลิตภัณฑ์สมุนไพรได้

Key words: Bar-HRM; DNA barcoding; Herbal pharmacovigilance; High Resolution Melting; Medicinal plants

ABSTRACT

Acanthaceae is a family of herbaceous plant existing throughout tropical and subtropical areas consisting of about 3,450 species in 300 genera. Among these, many are recognized in traditional Thai medicine records and commercialized in various processed forms posing problem in authenticity. Here, we evaluate the feasibility of technique Barcode DNA High Resolution Melting Analysis (Bar-HRM) in species identification of medicinal plants which is rapid, sensitive, and requires no taxonomical expertise. In doing so, two main parts of experiment were set up. First popular barcoding regions; ITS2 (from nuclear genome), *matK, psbA-trnH, rbcL* and *trnL* (from chloroplast genome) of medicinal plants were downloaded from GenBank and sequence profiles were then compared in order to address the most suitable marker for Bar-HRM analysis. Our results show that, in single locus analysis the *rpoC*1 primer set gave the highest discrimination (58%), and in multi locus analysis this could be increased from 87 – 99% depending on the total number of regions included. Different combinations proved to be more or less effective at discrimination, depending on the genus or family examined. In addition, *in silico* analysis suggested that internal transcribed spacer 2 (ITS2) exhibiting the highest nucleotide variability (88.12%) and richest GC content (66.79%), is thus likely to be the most suitable marker for Bar-HRM in discrimination of plant species in Acanthaceae. Second part of the experiment is to use the developed Bar-HRM method in authenticating commercial herbal products sold on markets. Ten "Fah Talai Jone" (*Andrographis paniculata*) products were included in the test and the analysed results indicated that all tested products contain "Fah Talai Jone" as promise on the product labels. However, the test of local products without proper packaging of three commonly used Acanthaceae species found that of all fifteen test samples, there are three of tested products did not contain the indicated species. Thus, the developed technique (Bar-HRM) was proved useful in species identification which demonstrates the robustness of this technique for application in herbal authentication.

Keywords: Bar-HRM; DNA barcoding; Herbal pharmacovigilance; High Resolution Melting; Medicinal plants

EXECUTIVE SUMMARY

Traditional medicine has played an important role in many South Asian countries including Thailand, and continues to do so today. Herbal products are a primary source of alternative medicines for the developed-world population (around 80%), yet quality control for raw plant material sold is very limited. In Southeast Asia, due to lack of cultivation of medicinal plants, the bulk of raw material is commonly collected from the wild with no provenance. Roots, bark, twigs, leaves, flowers, and seeds are sold under common names in local languages, so the potential for misidentifications and mixed collections is high. Authentication of herbal medicinal products on the herbal market becomes complicated because the original identifying characteristics (e.g. morphological characters) are absent in the majority of herbal products as they are mainly in the processed forms such as capsules, tablets, and powders. It is undeniable that quality and efficacy of herbal medicines are directly linked to the quality of the raw materials in which the consequences of species admixtures or adulteration can range from reducing the efficacy of the drug to lowering the trade value, not to mention accidental poisoning. An appropriate measure or a reliable method for species identification or authenticating of medicinal plant products is therefore urgently needed. In this study, we propose the use of new developed hybrid technique called Bar-HRM in aiding both taxonomical identification and authenticating herbal product sold on markets. Three main objectives were all achieved. First, we evaluate the most suitable DNA barcode region for species identification of medicinal plants, results of this part were in manuscript entitle "Evaluation of DNA barcoding coupled high resolution melting for discrimination of closely related species in phytopharmaceuticals" which has been submitted for peer review in an international journal. Second, we describe original work and provide novel data on the authentication of *Andrographis paniculata* "Fah Talai Jone" commercial products. *A. paniculata* is an important medicinal plant with multiple pharmacological properties. Results of this second part were in manuscript entitle "Hybrid Analysis (Bar-HRM) for Authentication of Thai Herbal Products, *Andrographis paniculata* (Burm.f.) Wall. ex Nees" which has been submitted for peer review in an international journal. Lastly, authenticating Thai local herbal product, Bar-HRM hybrid method was used for identifying medicinal plant species and authenticating of three commonly used herbal products on local markets (Chiang Mai Province). Results of the second part were already published (PLoS ONE. 05/2015; 10(5):1-11. DOI: 10.1371/journal.pone.0128476).

In conclusion, the method described in this study not only would be useful for species identification but also authentication of herbal products so that both traders and consumers would ultimately gain trust and confidence as there is a reliable way to authenticate the products regardless of their form (Herbal products are commonly sold in processed forms like powder and dried parts).

RESULTS AND DISCUSSION (เนื้อหางานวิจัย)

PART ONE:* *EVALUATION OF DNA BARCODING COUPLED HIGH RESOLUTION MELTING FOR DISCRIMINATION OF CLOSELY RELATED SPECIES IN PHYTOPHARMACEUTICALS

1.1 *DNA mining and primer design*

GenBank accessions were mined to assemble DNA barcodes of medicinal plants that are difficult to identify either as fresh materials or in their processed forms. Data was present for most markers of the 96 target species, except for *rpo*C1. The total number of sequences retrieved for the respective markers were: *rbc*L 79 of 96 species (82.3%); *mat*K 78.1%, *trn*L 76.0%, and *rpo*C1 1.0%. The absence of *rpo*C1 sequences for the target species was resolved by selecting 60 random *rpoC1* sequences from GenBank for primer design, which is supported by the high universality of *rpoC1* (Kress et al., 2005; Kool et al., 2012). The sequence data for the 96 medicinal plant species were extracted and aligned in order to create six universal primer sets for identification through high resolution melting (HRM) analysis.

A single set of primers was designed for each of *mat*K, *trn*L, and *rpo*C1, and three sets of primers were designed for *rbc*L, which yielded amplicons ranging from 100 to 150 bp. Reed and Wittwer (2004) found that amplicons suitable for HRM analysis should be 300 bp or less for optimal results. The *trn*L and *mat*K primer sets yielded amplicons of variable length with high standard deviation (SD) values, whereas the *rbc*L and *rpo*C1 primers set yielded amplicons of consistent size (Table 1.1).

Both the sequence length and the nucleotide variation within sequences influence the dissociation energy of the base pairs and result in different T_m values. The *mat*K and *trn*L amplicon sequences were observed to have higher nucleotide variation than the amplicons of the other regions, at 76.7% and 68.8%, respectively. The relative nucleotide variation within amplicons was found to be as follows: *mat*K > *trn*L > *rpo*C1 > *rbc*LB > *rbc*LC > *rbc*LA (Table 3). The forward and reverse *mat*K primers matched the consensus sequence of the target species at the binding sites in only 4 out of 24 sites (15.38%) and 10 out of 23 sites (43.48%), respectively (Table 1.1). High universality at the initial bases of the primer site is crucial for primer annealing and subsequent elongation initiation by the DNA polymerase.

Table 1.1 Characteristics of sequences and derived designed primers for high resolution melting analysis

Markers	rpoC1	trnL	rbcLA	rbcLB	rbcLC	matK
Available species/total (%)	60	28/96 (29.17)		53/96 (55.21)		47/96 (48.95)
Average total product, excluding primer (SD)	150, 108 (0)	97, 48 (8.93)	100, 55 (0)	149, 102 (0)	145, 99 (0)	158, 109 (4.3)
Characters (bp)	108	77	55	102	99	129
Variable characters (%)	58 (53.70)	53 (68.83)	19(34.54)	43(42.16)	38(38.38)	99(76.74)
Average distance (SE)	0.1145 (0.0175)	0.0358 (0.0194)	0.1137 (0.0316)	0.1250 (0.0222)	0.1179 (0.0229)	0.2968 (0.0376)
Conserved forward primer/total (%)	12/21 (57.14)	20/21 (95.24)	20/23 (83.96)	18/22 (81.82)	22/25 (88)	4/26 (15.38)
Conserved reverse primer/total (%)	16/21 (76.19)	25/27 (92.59)	18/22 (81.82)	22/25 (88)	16/21 (76.19)	10/23 (43.48)
Average %GC content (SD)	46.42 (1.93)	30.99 (6.81)	59.61 (2.85)	44.42 (2.04)	41.85 (1.46)	35.02 (3.02)

The *mat*K locus is one of the most variable plastid coding regions and has high interspecific divergence and good discriminatory power. However, it can be difficult to amplify with the standard barcoding primers due to high substitution rates at the primer sites (CBOL Plant Working Group, 2009; Hollingsworth, 2011). The *trn*L primer pair designed in this study was expected to be a suitable primer for HRM analysis for discrimination between the tested plant species. These *trn*L primers were nearly identical in base similarity to the mined consensus sequence, and the primer binding sites were designed in the conserved Q and R regions in the secondary structure of tRNA leucine (UAA) to optimise amplification success (Figure 1.1) (Taberlet et al., 2007). The *trn*L amplicon included the variable P6 loop in order to increase differentiation among the tested species during HRM. High primer binding success rates, short amplicon length, and high sequence variation make *trn*L an ideal marker for HRM based species discrimination in plants. The combination of these three characteristics has also made *trn*L the marker of choice for ancient DNA and ancient sedimentary DNA metabarcoding studies (Jørgensen et al., 2012; Parducci et al., 2012; Taberlet et al., 2012; Boessenkool et al., 2014; Willerslev et al., 2014). Recently, the application of *trn*L barcoding coupled with HRM has successfully detected adulteration in agricultural products (Madesis et al., 2012), has been used in forensics (Madesis et al., 2013), and has been used for taxonomic identification as well as hybrid tests (Ganopoulos et al., 2013).

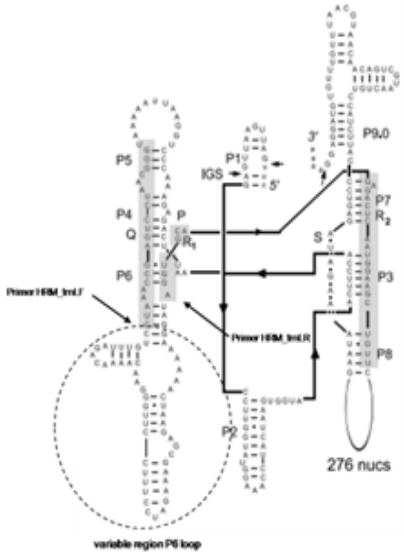


Figure 1.1 Partial structure of the rRNA of *trnL*. The regions shaded in grey indicate the primer binding sites.

The average %GC content of amplicons was calculated in order to predict variation in melting curves for the different markers. *trn*L had the lowest average %GC content, with 30.99%, followed by *mat*K, *rbcL*C, *rbcL*B, *rpo*C1 and *rbcL*A, with 35.02, 41.85, 44.42, 46.42 and 59.61% respectively (Table 1.1). The standard deviations (SD) values of the %GC content in each amplicon from the different primers reflect the wide sequence variation among the target species.

1.2 *In silico* amplicon identification rates

An *in silico* PCR for each primer pair was performed to test sequence variation and putative HRM species discrimination rates (Ficetola et al., 2010). The resolution of each HRM barcode at different taxonomic ranks was analysed (Figure 1.2). The input sequence file contained 231 sequence records representing the same number of species. The *mat*K primer amplicon could unambiguously identify only 43 of the 231 taxa from the taxonomic database. This low discrimination rate, in combination with the poor primer fit, indicate that *matK* is unsuitable for HRM analysis. Conversely, *trn*L gave the best rate of species level discrimination using HRM curves for identification (115 out of 231). This indicates that *trn*L is a good candidate for HRM due to: (i) high SD within amplicons lengths; (ii) high rate of character variation; (iii) high conservation in the primer sites in the consensus sequence; and (iv) high SD of the average %GC content.

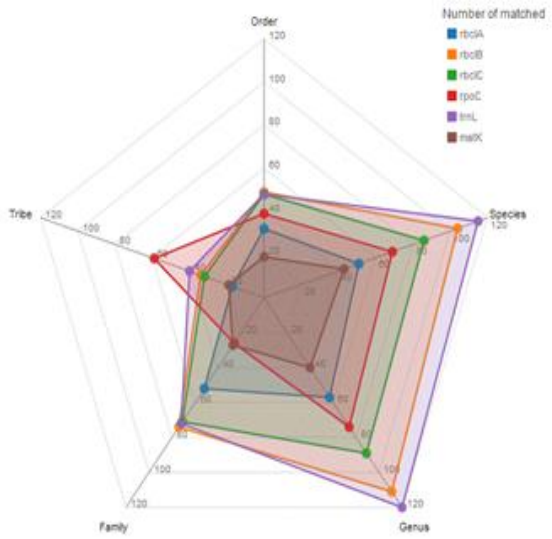


Figure 1.2 Results from *in silico* PCR of each primer pair. The resolution of each HRM barcode on the chloroplast genome at different five taxonomic ranks including Order, Tribe, Family, Genus and species are shown. Testing is based on 231 sequence records retrieved from GenBank.

1.3 *Evaluation of amplicons for medicinal plant species discrimination by Bar-HRM*

The six primers pairs designed to amplify sections of four chloroplast markers were tested with 26 medicinal plant species from 14 families using HRM analysis (Table 1.2). These six primer sets amplified products from *mat*K, *rbc*LA, *rbc*LB, *rbc*LC, *rpo*C1, and *trn*L and yielded amplicons of 170 bp, 100 bp, 150 bp, 150 bp, 170 bp, and 120 bp, respectively. HRM analysis was performed in triplicate on each of the 26 taxa to establish the T_m for each primer set. The shapes of the melting curves were analysed using EcoStudy Software v 5.0 to distinguish the different plant species. Bar-HRM species identification success rates were assessed for each single-locus as well as for multi-locus combinations to establish the optimal combination of primer sets.

Table 1.2 Plants included in this study are all commonly found in Thai markets.

Family	**Scientific name** [a]
Acanthaceae	*Acanthus ebracteatus* Vahl, *Andrographis paniculata* (Burm.f.) Nees
	Barleria lupulina Lindl., *Clinacanthus nutans* (Burm.f.) Lindau
	Rhinacanthus nasutus (L.) Kurz, *Thunbergia laurifolia* Lindl.
Araliaceae	*Schefflera leucantha* R.Vig.
Compositae	*Pluchea indica* (L.) Less.
	Cyanthillium cinereum (L.) H.Rob.
Cucurbitaceae	*Momordica charantia* L.
Euphorbiaceace	*Phyllanthus amarus* Schumach. & Thonn.
Lamiaceae (Labiatae)	*Orthosiphon aristatus* (Blume) Miq.
Leeaceae	*Leea macrophylla* Roxb. ex Hornem.
Leguminosae	*Senna siamea* (Lam.) H.S.Irwin & Barneby
	Senna alata (L.) Roxb.
Menispermaceae	*Tinospora crispa* (L.) Hook.f. & Thomson
	Tinospora sinensis (Lour.) Merr.
Moringaceae	*Moringa oleifera* Lam.
Papilioneae	*Derris scandens* (Roxb.) Benth.
Piperaceae	*Piper sarmentosum* Roxb.
Vitaceae	*Cissus quadrangularis* L.
Zingiberaceae	*Amomum verum* Blackw., *Boesenbergia rotunda* (L.) Mansf.
	Curcuma longa L., *Curcuma zedoaria* (Christm.) Roscoe.
	Zingiber montanum (J.Koenig) Link ex A.Dietr., *Zingiber officinale* Roscoe
	Zingiber ottensii Valeton, *Zingiber zerumbet* (L.) Roscoe ex Sm.

[a] Plant nomenclature following The Plant List (www.theplantlist.org)

1.3.1 Single-locus analyses

The HRM primers sets were used for the amplification of DNA-fragments from all 26 Thai medicinal plant species, and the resulting amplicons were analysed using HRM to define T_m. The melting profiles of all amplicons are illustrated in Figures 1.3 and 1.4. The analysis is presented by means of conventional derivative plots, which show that the T_m value of each species is represented by a peak. The melting temperature peaks of all the 26 medicinal plant species are calculated as T_m and presented in Supplementary Data 1.

The mean of the melting temperatures obtained from each primer pair was used to measure species discrimination for each locus. These species discriminations for each locus ranged from 15% (*mat*K) to 58% (*rpo*C1), with *trn*L, *rbc*LA, *rbc*LB, and *rbc*LC, providing 40%, 32%, 32%, and 28% discriminatory power, respectively (Figure 5). Although *mat*K has been proposed as one of the best plant barcodes in terms of species discrimination (Fazekas et al., 2008; Hollingsworth et al., 2009) we found that the section of *mat*K amplified by our novel primer set for HRM has a low success rate in PCR amplification and also a low species discrimination rate among the taxa that were successfully amplified. Conversely, the high species discrimination for *rpo*C1 (58%), contrasts with previous barcoding studies that indicate that this marker has relatively low discriminatory power (Fazekas et al., 2008; Hollingsworth et al., 2009; Kool et al., 2012). Not all amplicons from the different species yielded distinctive HRM profiles (Figure 3), but all could be discriminated at the family level, with the exception of *mat*K. Furthermore, each primer set gave different rates of species discrimination in different families. For example, none of the primer sets could clearly distinguish among species in Zingiberaceace. However, the *rbc*LB primer pair enabled division of Zingiberaceace into two groups: (i) *Curcuma longa* L. and *Curcuma zedoaria* (Christm.) Roscoe and (ii) *Zingiber montanum* (J.Koenig) Link ex A.Dietr., *Zingiber officinale* Roscoe and *Boesenbergia rotunda* (L.) Mansf. (Figure 6). Zingiberaceae is known to be a difficult group to identify using molecular methods, and recent studies have shown that the nuclear ribosomal marker ITS2 is the best single marker for species identification (Shi et al., 2011; Chen et al., 2014). Other families were easier to identify, such as the family Acanthaceae, which in this dataset had a high rate of species discrimination for two of the loci, *rpo*C1 (66.7%) and *trn*L (50%) (Figure 1.5).

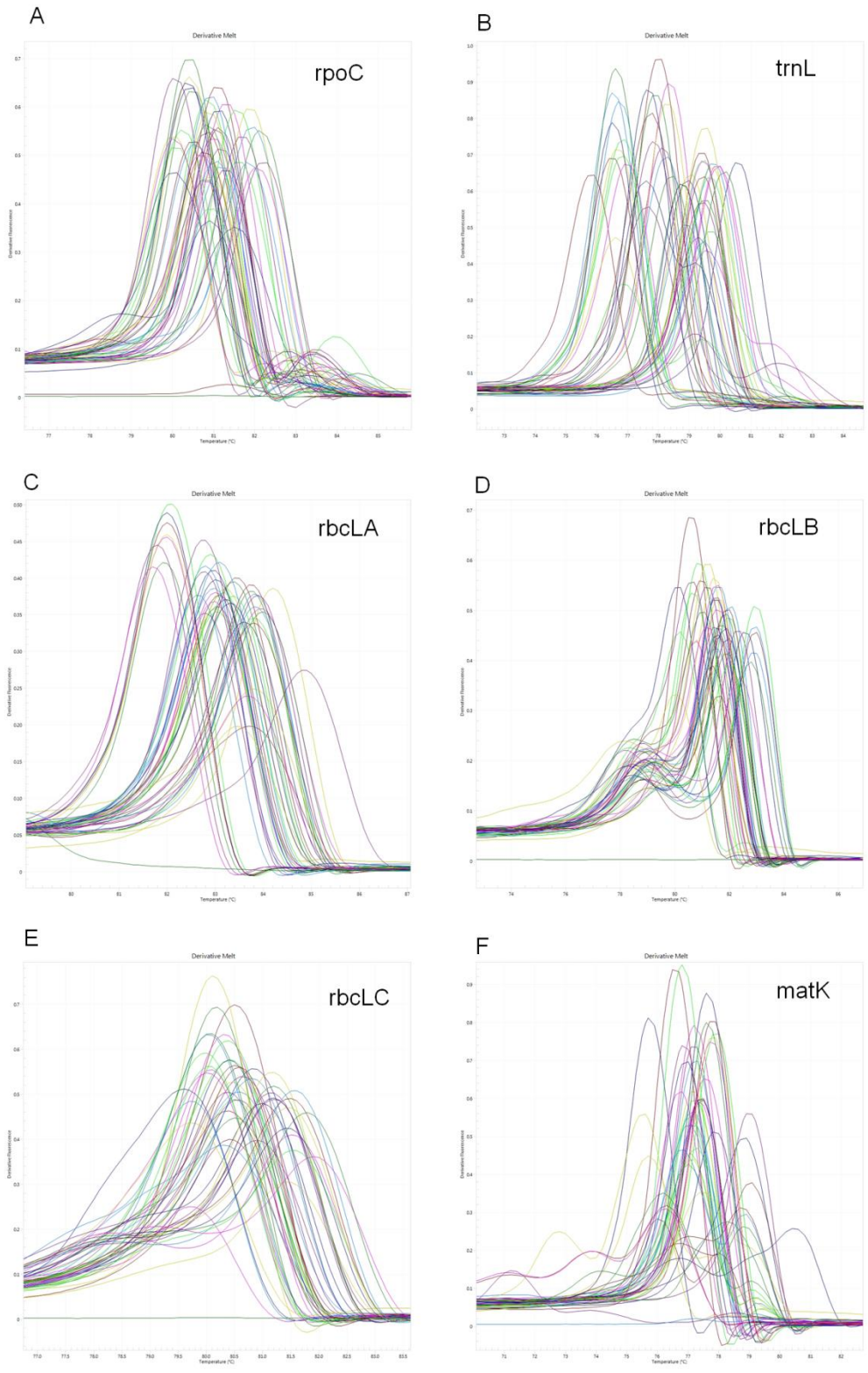


Figure 1.3 Melting curve profiles of amplicons obtained from each primer set: *rpoC*1 (A), *trnL* (B), *rbcL*A (C), *rbcL*B (D), *rbcL*C (E) and *matK* (F).

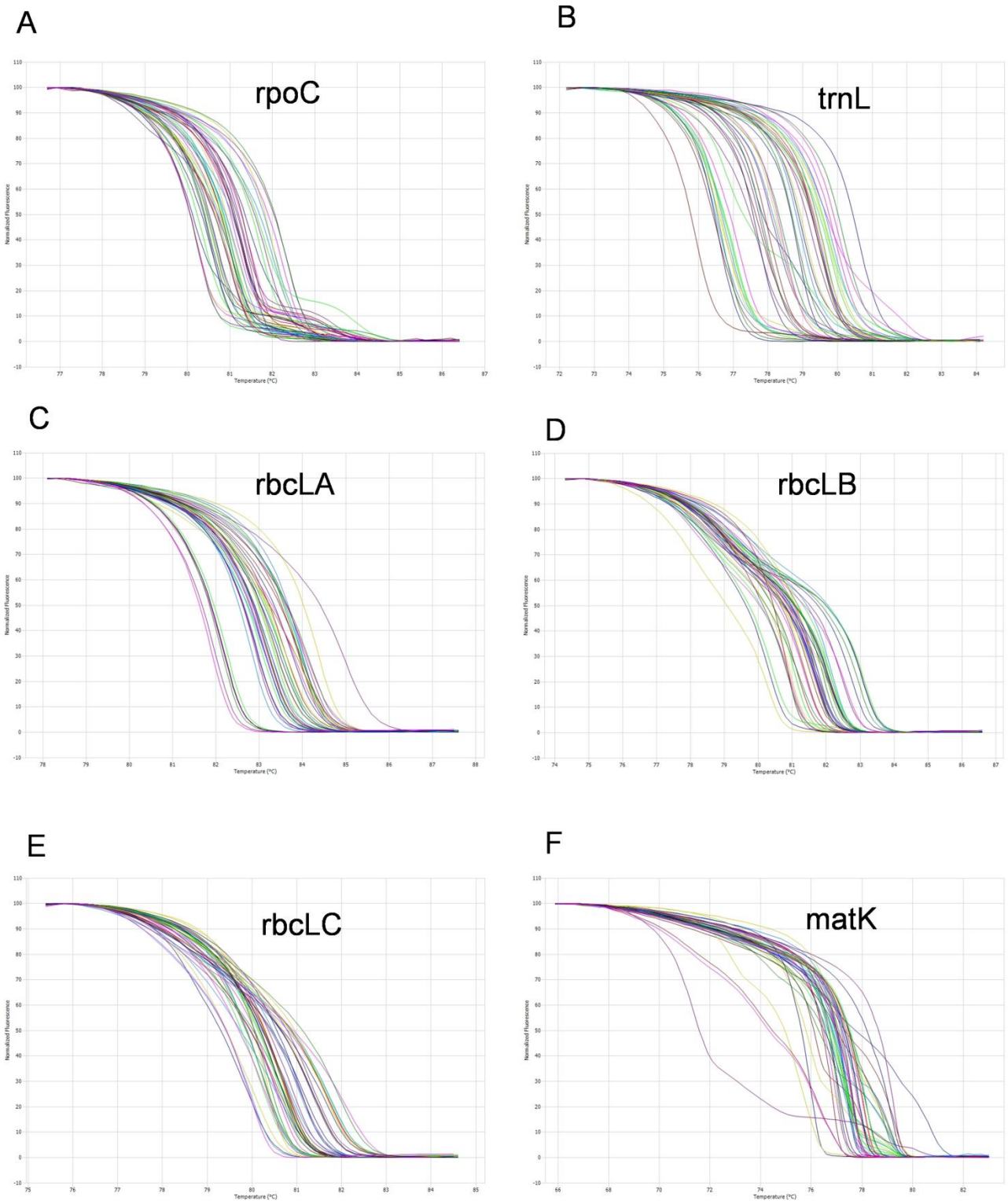


Figure 1.4 The normalised plot of each primer pair derived from four barcode regions shows the differentiation of melting temperature (T_m) of each amplicon from each species, generated by high resolution melting (HRM) analysis.

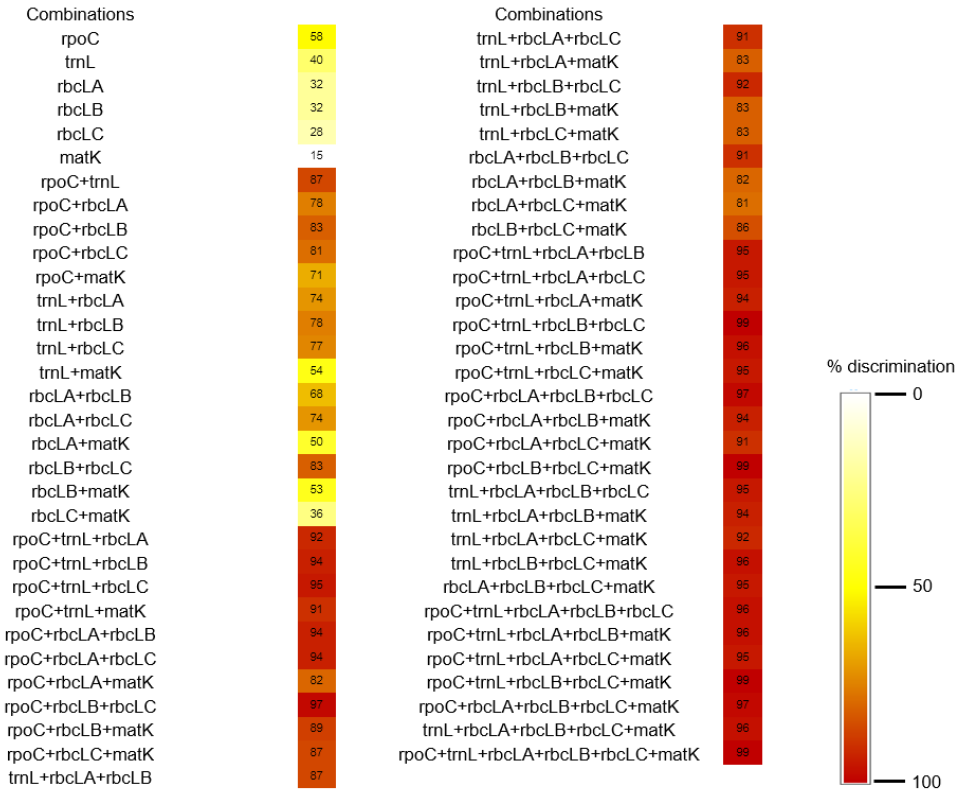


Figure 1.5 Discriminatory power of six loci from four barcoding regions (*rbcL*, *trnL*, *rpoC*1 and *matK*) for species identification using high resolution melting (HRM) analysis and their combinations (2-6 combinations) expressed as percentage of accurately discriminated species.

Results from the present study are similar to those from previous studies suggesting that each single marker has limited resolution and that combinations of two or more markers are needed for plant species identification (Hollingsworth, 2011). Here we find that no single marker identifies more than 58% of all species (*rpo*C1). Nevertheless, it could be that other single markers suggested by the results of other studies, such as ITS1, ITS2, *trn*H-*psb*A, could be used to produce HRM primer sets with even higher resolution (Kress et al., 2005; Fazekas et al., 2008; Chen et al., 2010; Gao et al., 2010; Li, Gao, et al., 2011; Kool et al., 2012; de Boer et al., 2014).

1.3.2 Multi-locus analyses

Simultaneously employing multiple standard loci to identify taxa, a method known as multi-locus DNA barcoding, reduces the overall negative effects of lacking amplicons due to variable primer-sites within taxa, while it increases discriminating power in plant identification.

The use of multi-locus DNA barcoding has been recommended from the inception of barcoding as a method for identification in plants (Kress et al., 2005; Kress and Erickson, 2007; Fazekas et al., 2008; CBOL Plant Working Group, 2009). For example, Kress et al. (2005) recommended combining ITS and *trn*H-*psb*A, Kress and Erickson (2007) recommended *trn*H-*psb*A and *rbc*L, Fazekas et al. (2008) evaluated eight different plastid markers, and the CBOL Plant Working Group (2009) evaluated seven plastid markers and recommended a combination of *rbc*L and *mat*K, possibly supplemented with ITS (Hollingsworth, 2011). To find an optimal combination of HRM primer sets all combinations of the six studied loci were evaluated, in relation to the differences among the obtained T_m values. The discriminatory power of the primer sets was tested using discriminant analyses.

In single locus analysis the T_m profiles from the *rpo*C1 primer set gave the highest discrimination (58%), and in multi locus analysis this could be increased from 87 – 99% depending on the total number of regions included. When using the two primer pairs *rpo*C1 + *trn*L discrimination reached 87%, and with three pairs 95% and 97% discrimination could be made with *rpo*C1 + *trn*L + *rbc*LC and *rpoC1* + *rbc*LB + *rbc*LC, respectively. In four loci analyses species resolution ranged from 91% (*rpo*C1 + *rbc*LA + *rbc*LC + *mat*K) to 99% (*rpo*C1 + *trn*L + *rbc*LB + *rbc*LC and *rpo*C1 + *rbc*LB + *rbc*LC + *mat*K). The success of the species resolution reached an upper limit of 99% with four markers, and this was not further improved with five or six markers (Figure 1.5). Due to the low universality of the forward *mat*K primer (HRM_matK1F), we recommend the use of the *rpo*C1 + *trn*L + *rbc*LB + *rbc*LC combination over the *rpo*C1 + *rbc*LB + *rbc*LC + *mat*K combination.

In this study, identification of all Zingiberaceae species was not possible, even when using data from all six loci. Among the Acanthaceae species, a combination of *rpo*C1 and *trn*L identified 94.4% of species, whereas the combination of *rpo*C1 and *rbc*LA identified 100% of species. These results indicate that taxa in different plant groups may be more or less readily discriminated by different combinations of markers. Thus, when identification is focused on specific families or genera is it essential to determine the optimal combination of markers for species discrimination. These marker combinations can be estimated based on available sequence data in public repositories, and used for the design of unique and reliable HRM primer sets for species identification.

PART TWO: HYBRID ANALYSIS (BAR-HRM) FOR AUTHENTICATION OF THAI HERBAL PRODUCTS, ANDROGRAPHIS PANICULATA (BURM.F.) WALL.EX NEES

2.1 DNA barcodes data and primer pair

In order to develop a new method combining the DNA barcoding and HRM analysis for discrimination of *A. paniculata* and other related species and also its further use for the authenticating the species products on the market, availed DNA barcodes data on the online databases were collected. From the alignment of the extracted data (Figure. 2.1A), the primer pair named rbcL_F and rbcL_R was chose for HRM analysis as products expected from this primers contained nucleotides variation of the different species which would allow their discrimination. The sequences of the species tested were used to construct phylogenetic tree (Figures. 2.1B).

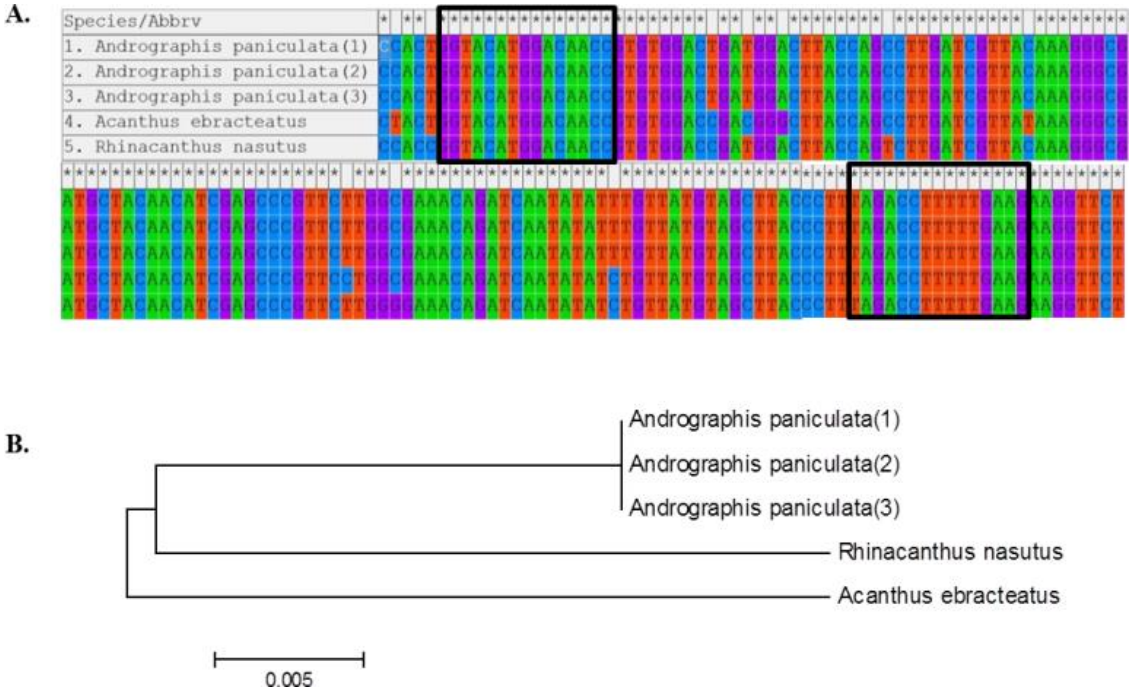


Figure 2.1 Comparative alignment of *rbc*L consensus DNA barcodes of *A.paniculata, A. ebracteatus* and *R.nasutus* and phylogenetic tree using MEGA5.

(**A**) Common DNA barcodes in three Acanthaceae were generated based on DNA sequences obtained from available DNA database sequences information from NCBI for *rbc*L region. The boxes denote the region were the primer pair was designed. (**B**) Phylogenetic NJ tree based on DNA barcodes derived from DNA database sequences information from NCBI for *rbc*L region (Accession numbers of each sample, *A.paniculata* ; JF949965.2, JQ922118.1 and JQ230990.1, *A.ebracteatus*; AY289682.1, *R.nasutus*; GQ436493.1).

A real-time PCR protocol was applied for the identification and quantitative determination of *A. paniculata*in commercial herbal products. Table 2.1 depicts the results ofthe SYBR assay for the discrimination of the *A. paniculata* and related species. Herbal samples from different species could be distinguished using HRM analysis and the designed primers *rbc*L. The melting profiles of the *rbc*L amplicons of the three closely related herbal species (*Andrographis paniculata, Acanthus ebracteatus* and *Rhinacanthus nasutus*) are illustrated in Figures. 2.2A and 2.2B. A distinct melting curve was generated for each herbal species presenting one inflection point. Analysis of the normalised HRM curves with the barcode marker *rbc*L (Figures. 2.2A and 2.2B) revealed that the three species could easily be distinguished. Assigning species *A. paniculata* as a genotype we were able by subtracting the area (difference graph) from the rest of the produced melting curves by the other species, to estimate the confidence value of similarity between the three species used (Figure. 2.2B). GCPs were calculated and a cut off value of 90% was used to assign a genotype for each barcode region. Furthermore, a closer examination of the *A. paniculata* HRM difference curve, with the mean of *A.ebracteatus* and *R. nastus* with *A. paniculata* curve as the baseline, revealed part of the curve sitting outside the 90% CI curve, suggesting that the *A. ebracteatus* and *R. nastus* HRM curves are indeed different (Fig. 2.2B). The HRM analysis with the designed primer pairs proved to be a powerful tool in the identification of more or less closely related Acanthaceae species. The reproducible individual melting curves were achieved from different species with triplicate. Similar melting curves were achieved from the same species regardless of the type of the DNA template (fresh or dried tissue, leaf, and stem) (data not shown). Thus, the method can be applicable also for the processed herbal products that still contain tissue material with intact DNA.

Table 2.1 The values of melting temperature (˚C) gaining for high resolution melting (HRM) analysis using *rbc*L markers of *Andrographis paniculata* and related species

species	**T_m (˚C)**
Andrographis paniculata	81.15±0.06 81.66±0.03 80.10±0.06

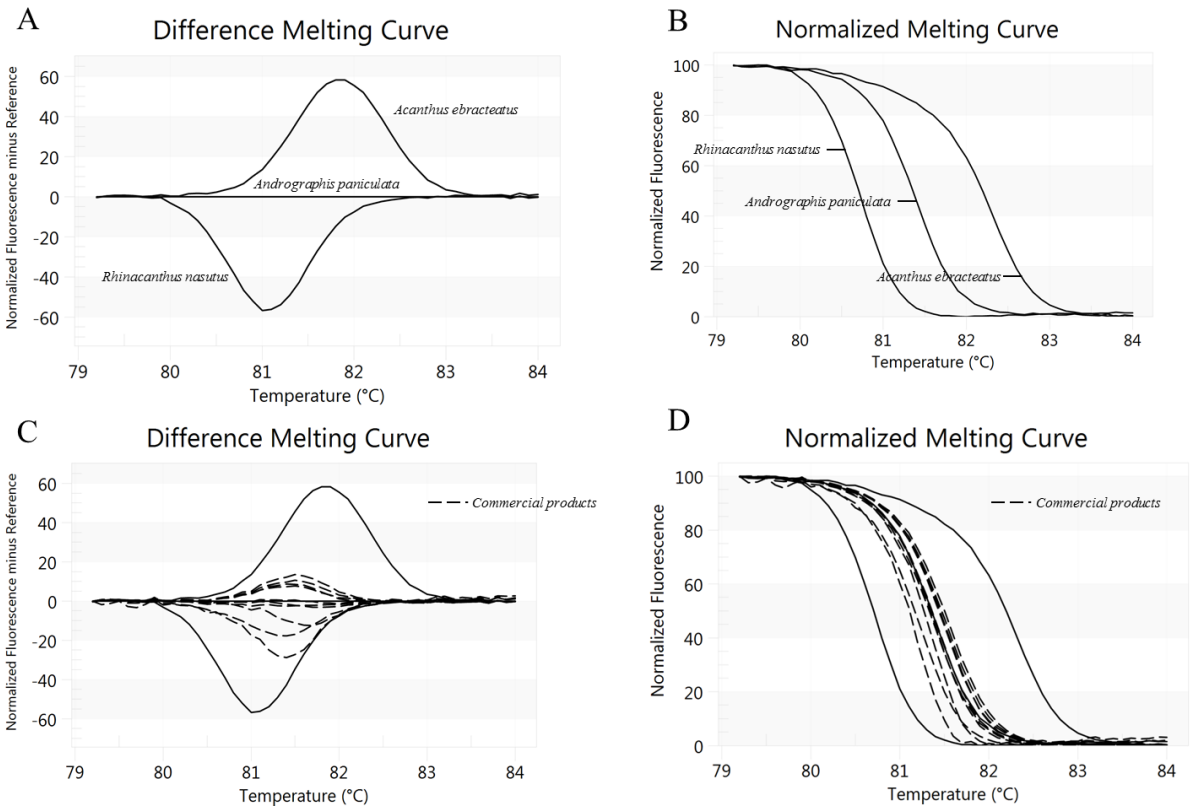


Figure 2.2 Barcoding of three species tested using HRM analysis with the designed *rbc*L chloroplast marker. (**A**) Difference graph of the three species using *A.paniculata* as reference. (**B**) Melting curves of the *rbc*Lamplicons from the three species using *A.paniculata* as reference. HRM analysis using the designed *rbc*L primers analysis of ten commercial products labelled as *A. paniculata*. (**C**) Normalised curves of the three species *A.paniculata, A.ebracteatus, R.nasutus* and ten commercial products. (**D**) Melting curves of the *rbc*L amplicons from the three species and ten commercial products.

2.2 Detection of A. paniculata in herbal products

After the confirmation that each tested species (three Acanthaceae species of which *A. paniculata* was used as references) can be identified by HRM analysis we applied the same approach for the identification of the species used in herbal products (Table 2.2). The DNA extracted from all products tested yielded a specific amplification product with the *rbc*L primers. The normalised HRM curves for the amplicons, from the species studied and 10 commercial ‘‘Fah Talai Joan’’ herbal products, based on HRM analysis with barcode marker *rbc*L are shown in Figures 2.2C and 2.2D. The products tested produced a unique melting plot that was easily to

spot, thus all commercial samples could be successfully assigned to the species. All 10 products contained the *A. paniculata* or ‘‘Fah Talai Joan’’ that was promised or labelled as can be seen from their normalised HRM curves (Figures 2.2C and 2.2D). Furthermore, closer examination of the HRM difference curve, with all the tested samples with *A. paniculata* curve as the baseline, revealed part of the curve sitting outside the 90% CI curve, only for *A. ebracteatus* and *R. nasutus* suggesting that the *A. ebracteatus* and *R. nasutus* HRM curves are indeed different and all the other samples tested are similar to *A. paniculata*. Thus DNA barcode coupled with HRM analysis methodology has allowed us easily to determine the *A. paniculata* species in herbal products on the markets even they are in processed forms.

Table 2.2 Commercial *A. paniculata* or FahTalai Joan products and related species used in this study.

Species/type	Source	Form
Andrographis paniculata	QSBG voucher no. 68296	Dried tissue
Acanthus ebracteatus	QSBG voucher no. 29333	Dried tissue
Rhinacanthus nasutus	QSBG voucher no. 63282	Dried tissue
Andrographispaniculata	Medicinal Plant Garden	Fresh leave
Acanthus ebracteatus	Medicinal Plant Garden	Fresh leave
Rhinacanthus nasutus	Medicinal Plant Garden	Fresh leave
Commercial 1	Online market	Capsule
Commercial 2	Market	Capsule
Commercial 3	Market	Capsule
Commercial 4	Market	Capsule
Commercial 5	Market	Tablet
Commercial 6	Market	Capsule
Commercial 7	Market	Capsule
Commercial 8	Market	Capsule
Commercial 9	Market	Capsule
Commercial 10	Local producers	Powder

This is partly indicated that the regulations of quality control in the herbal industry are seem to be appropriate and rigorous in Thailand, although we could not conclude this for other herbal products. In Thailand *A. paniculata* is one among popular species used by a vast number of people. Luckily, misidentification has not been an issue for *A. paniculata* species but several medicinal plants of Thailand are currently facing the issue. The method developed in this study

would be useful for authentication of others products too. Both traders and consumers would ultimately gain trust and confidence as there is a reliable way to authenticate the products. The developed method could be improved further if it is used along with a more species-specific primer and more species tested, which has been demonstrated for resolving individuals contributing trace amounts of DNA to highly complex mixtures.

PART THREE: ***BAR-HRM FOR AUTHENTICATION OF PLANT-BASED MEDICINES: EVALUATION OF THREE MEDICINAL PRODUCTS DERIVED FROM ACANTHACEAE SPECIES***

3.1 Data mining and primers used

The amplification of the *rbc*L locus from three medicinal plant species in this study was performed using specific primers corresponding to the *rbc*L barcode region. All *rbc*L sequences of Acanthaceae were extracted from GenBank and the variable characters, average distance, and average %GC content were calculated for all samples using MEGA6. In total, 248 sequences were retrieved, of which 235 sequences were deemed useful for further analysis (Table 3.1). An alignment of all useful sequences was made, and a 136 nucleotide base-pair fragment was analyzed after its amplification with newly developed forward and reverse primers. Thirty-six variable sites (26.5%) and 41.2% GC content were observed within the fragment (Figure 3.1A). The average distance was calculated for each sequence and plotted using Gephi (Figure 3.1B). Figure 3.1B shows that all sequences from samples of the same genus cluster together. The majority of *rbc*L sequences are from *Justicia*. Commonly used Thai herbs belong predominantly to *Andrographis*, *Acanthus* and *Thunbergia*, though sequences from these genera are limited in our reference dataset. Among the total of 235 analysed sequences, we found only one mismatched nucleotide position on the reverse primer site, and a perfect match among all samples for the forward primer. Thus, these newly developed primers, which were designed based on the sequences extracted from GenBank, were predicted to perform well in HRM analyses with the medicinal plant species in question.

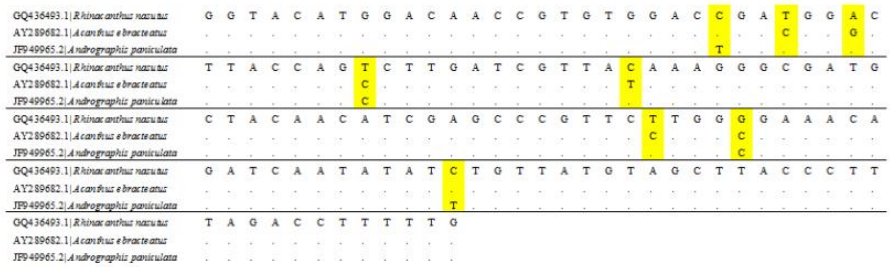
Table 3.1 Acanthaceae sequences of *rbcL* were retrieved from GenBank (NCBI) for each of the genus with accession number.

Genus	Sequence	Acession number (NCBI)	Genus	Sequence	Acession number (NCBI)
Acanthus	4	AY289682, L12592	*Ecbolium*	1	JQ933315
		HE963302, HM849737	*Echinacanthus*	1	JQ933316
Aechmanthera	1	JQ933209	*Elytraria*	1	AF188127
Andrographis	9	GQ436494-GQ436496	*Eranthemum*	4	JQ734505-JQ734506
		JQ230990, JQ922118			JQ933327, JQ933456
		JQ933217, JF949965	*Hypoestes*	3	AB586152-AB586153
		KF365996, KF425766			L12593
Anisotes	1	JF265288	*Hygrophila*	2	GU135241, GU135244
Aphelandra	20	GQ981668, L01884	*Isoglossa*	1	AM234780
		JQ590007-JQ590024	*Justicia*	62	JQ590033-JQ590068
Asystasia	3	GU135172, JQ933229			JQ231000, JX572702
		JQ673521			JQ594958-JQ594960
Avicennia	22	AY008829-AY008832			KC756924-KC756934
		AY289681, JX572318			KF669388-KF669393
		JQ590025-JQ590030			DQ006045, L01930
		JQ594360-JQ594364			GQ436497, GQ436500
		JQ594977-JQ594981			HM850082, L14401
		U28868	*Lepidagathis*	1	L12594
Baphicacanthus	2	GQ436498-GQ436499	*Mackaya*	1	JX572742
Barleria	16	L01886, JQ673524	*Metarungia*	1	JF265518
		AB586149-AB586151	*Monechma*	2	AM234781, AB586154
		JF265299-JF265300	*Nelsonia*	4	HQ384879, L01935
		JQ590031-JQ590032			JQ590069-JQ590070
		KF890169-KF890172	*Odontonema*	3	JQ590071-JQ590073
		JQ231001	*Peristrophe*	2	AM234782, KF425772
Blepharis	1	JQ673527	*Petalidium*	1	JQ933440
Chaetacanthus	1	AM234779	*Phaulopsis*	1	JQ933444
Clarkeasia	1	JQ933269	*Ruellia*	20	GU135168, GU135171
Clinacanthus	1	GQ436501			GU135266, L12595
Crossandra	1	JQ933287			AB586155-AB586156
Diceratotheca	1	JX469440			JQ673547-JQ673548
Dicliptera	1	JQ933303			JQ590074-JQ590085
Duvernoia	2	JF265402-JF265403	*Rhinacanthus*	2	KF381120, GQ436493

Table 3.1 (Continued) Acanthaceae sequences of *rbcL* were retrieved from GenBank (NCBI) for each of the genus with accession number.

Genus	Sequence	Acession number (NCBI)
Rungia	1	JQ933466
Ruspolia	2	JF265577
		JX572942
Ruttya	7	AB586157-AB586161
		JF265578
		L02434
Sanchezia	1	AJ247613
Sclerochiton	2	JX572957-JX572958
Strobilanthes	2	JQ933455
		JQ933492
Thunbergia	6	AM234783
		AY008828
		L12596
		HQ384878
		JQ590086
		KF181493
Trichanthera	1	GQ981903

A



B

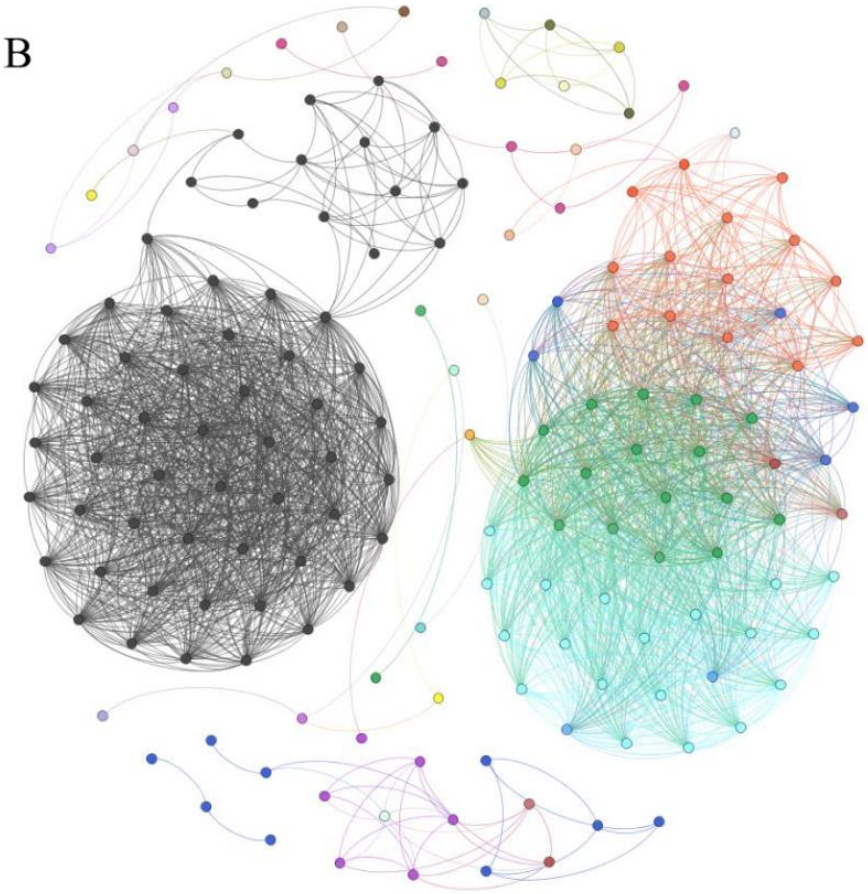


Figure 3.1 Variable sites and Distance plot of the sequences retrieved from GenBank. **A)** Nucleotide variation in the 136 bp *rbcL* fragment of 235 aligned Acanthaceae species. **B)** Gelphi distance plot of the *rbcL* sequences belonging to 47 genera of Acanthaceae downloaded from GenBank**.**

3.2 HRM analysis

The newly designed *rbc*L primer pair yielded amplicons of the expected size, approximately 136 base-pairs long. Eight variable sites were observed within the fragment of the three tested herb species (Figure 3.2A). The amplicons were analyzed using HRM to determine the T_m. Figures 3.2B and 3.2C present the analysis by means of conventional derivative plots, which show that the T_m value for the *rbc*L fragment from each species was represented by a peak. The melt curve is generated by slowly melting the DNA of tested plant species through a range of temperatures in the presence of a dsDNA binding dye. The melting temperature peaks of the tested plant species are calculated as T_m. HRM analysis with this primer pair proved to be a powerful tool for the identification of the three closely related Acanthaceae species (*Ac. ebracteatus*, *An. paniculata* and *R. nasutus*). The individual melting curves were reproducibly achieved from each of the three different species in triplicate analyses. Similar melting curves were achieved from the same species regardless of whether the DNA template was extracted from fresh or dried tissue (Figures 3.2B and 3.2C). Thus, the method may also be applicable for processed herbal products that still contain tissue material with intact DNA.

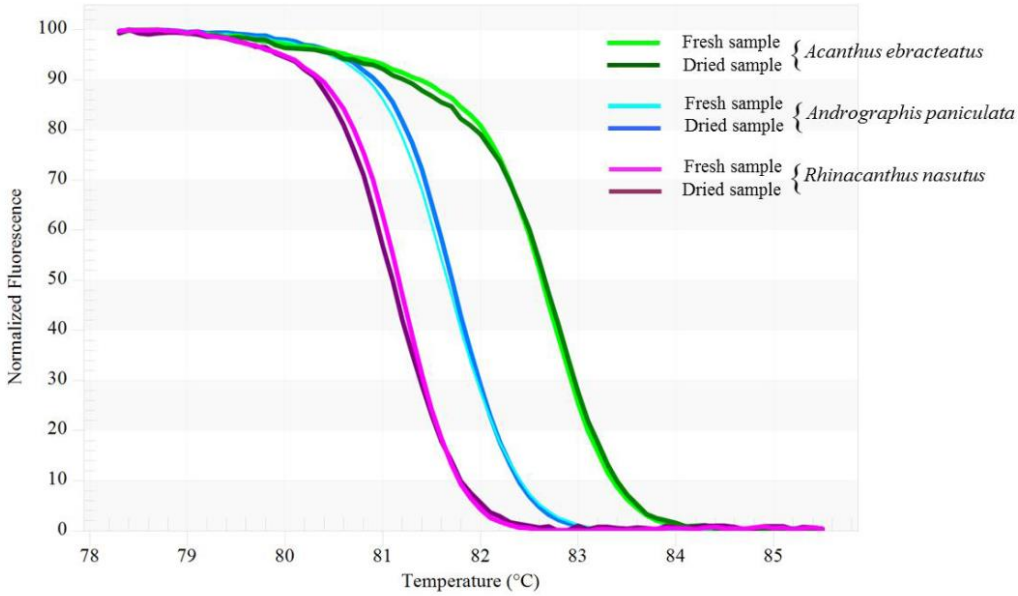
3.3 Identification of herbal species in local products

Constituent species in herbal products bought from local markets in Thailand were investigated to assess the reliability of information regarding their ingredients, as the herbal products are often sold without proper packaging or labeling. Fifteen herbal products were purchased from local producers. The HRM analysis using *rbc*L primers was then performed to identify the species in the products and to see whether they are consistent with the species indicated by the sellers. The results of the analysis reveal that two samples which sellers identified as *An. paniculata* were actually *R. nasutus* (products no. 11 and 14), while one which sellers identified as *R. nasutus* was actually *An. paniculata* (product no. 6) (Figure 3.3).

A

Base position	23	29	32	38	44	45	49	50	51	53	58	59	62	65	68	71	74	75
Nucleotide found	C	T	C	T	C	A	T	A	G	A	T	A	T	A	C	A	C	C
	T	C	T	C	G	T	A	G	C	G	G	G	G	G	T	T	A	T
Base position	**80**	**84**	**86**	**88**	**89**	**98**	**99**	**101**	**103**	**110**	**111**	**112**	**113**	**120**	**121**	**122**	**131**	**134**
Nucleotide found	T	C	A	G	T	T	C	G	G	T	C	C	T	G	T	T	C	A
	C	A	C	A	C	C	T	A	A	A	A	A	A	A	C	G	T	G
								T		C	G	T						

B



C

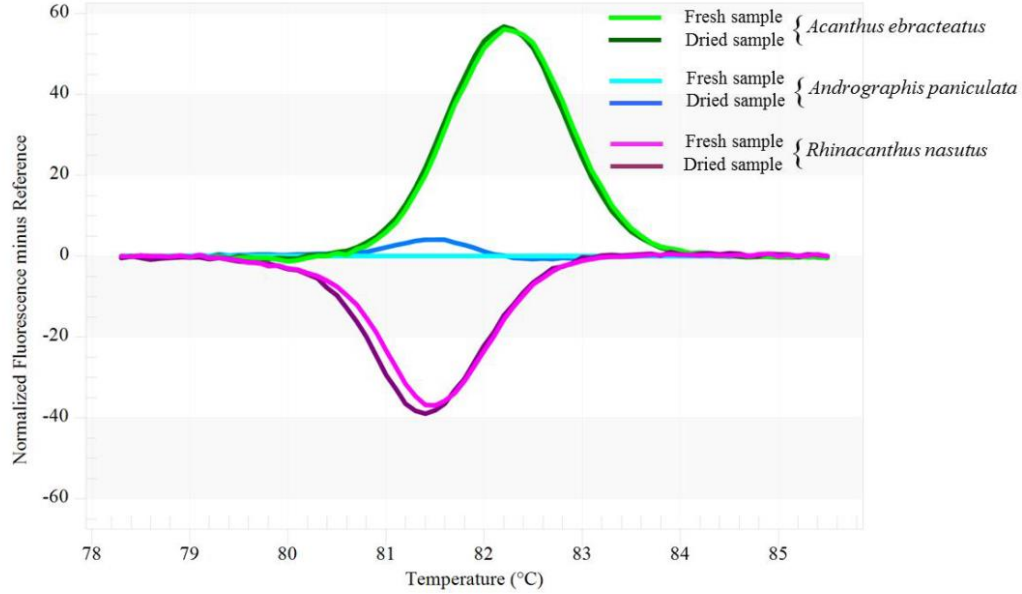


Figure 3.2 *rbcL* nucleotide alignment and melting profiles of the three Acanthaceae species. **A)** Variable sites detected in *rbcL* sequences of the three closely related species, *Acanthus ebracteatus*, *Andrographis paniculata* and *Rhinacanthus nasatus*. **B)** Normalised fluorescence plot of barcoding coupled high resolution melting (Bar-HRM). **C)** Reference-corrected normalised fluorescence plot of barcoding coupled high resolution melting (Bar-HRM).

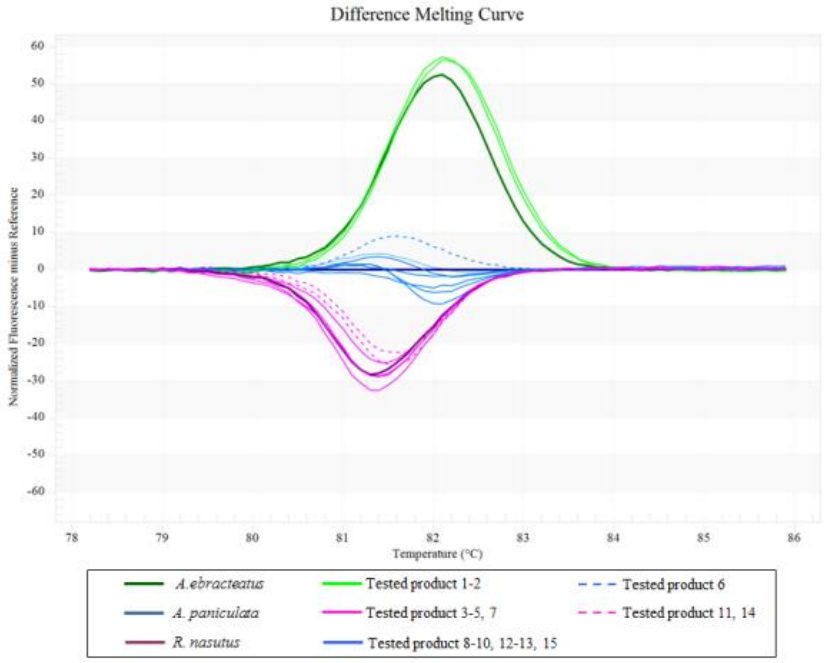


Figure 3.3 Representative profiles of the melting curves (difference plot curves). Bar-HRM difference plot curves obtained using the *rbc*L primer pair to identify 15 local herbal products. Three tested products (6, 11 and 14) were found to be different species from the species indicated by the vendor.

OUTPUT

1. Gaining a novel effective and relatively cheap method based on barcoding and high resolution melting (HRM) analysis for the routine authenticating and identification of medicinal raw materials and products on the market. The obtaining hybrid barcoding and HRM technique with a great efficiency in medicinal plant species discrimination in this project would be a fundamental tool to ease and ensure both quality and safety of herbal products and could be further applied for other groups of medicinal plant species.

2. A DNA barcode bank generated in this project could be a source of comprehensive medicinal plant species database which will be readily aid in identification of plant specimens.

3. Publications in an international scientific journal.

APPENDIX (ภาคผนวก)

1.1 **Maslin Osathanunkul***, Panagiotis Madesis, Hugo de Boer (2015) DNA data of commonly used medicinal plants from the family Acanthaceae which were used in primers designing. Four primer pairs were constructed and used in HRM analysis for identifying and authenticating herbal products. *PLoS ONE*. 05/2015; 10(5):1-11. DOI: 10.1371/journal.pone.0128476. (impact factor 3.53)

1.2 Sahachat Singtonat and **Maslin Osathanunkul*** (2015) Fast and reliable detection of toxic *Crotalaria spectabilis* Roth. in *Thunbergia laurifolia* Lindl. herbal products using DNA barcoding coupled with HRM analysis. *BMC Complementary and Alternative Medicine*.15:162 DOI 10.1186/s12906-015-0692-6. (impact factor 1.88) Joint funding of TRF and CMU Junior Research Fellowship Program. (but work done mainly on CMU grant)

1.3 **Maslin Osathanunkul***, Chatmongkon Suwannapoom, Danupol Pintakum, Santisuk Na Lamphun, Kanokporn Triwitayakorn, Panagiotis Madesis. Hybrid Analysis (Bar-HRM) for Authentication of Thai Herbal Products, *Andrographis paniculata* (Burm.f.) Wall.ex Nees. Submitted to *Pharmacognosy Magazine* (impact factor 1.525) in review process.

1.4 **Maslin Osathanunkul***, Chatmongkon Suwannapoom, Panagiotis Madesis, Hugo de Boer. Evaluation of DNA barcoding coupled high resolution melting for discrimination of closely related species in phytopharmaceuticals. Submitted to *Applications in Plant Sciences* in review process

PLOS | ONE

RESEARCH ARTICLE

Bar-HRM for Authentication of Plant-Based Medicines: Evaluation of Three Medicinal Products Derived from Acanthaceae Species

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

Medicinal plants are used as a popular alternative to synthetic drugs, both in developed and developing countries. The economic importance of the herbal and natural supplement industry is increasing every year. As the herbal industry grows, consumer safety is one issue that cannot be overlooked. Herbal products in Thai local markets are commonly sold without packaging or labels. Plant powders are stored in large bags or boxes, and therefore buying local herbal products poses a high risk of acquiring counterfeited, substituted and/or adulterated products. Due to these issues, a reliable method to authenticate products is needed. Here DNA barcoding was used in combination with High Resolution Melting analysis (Bar-HRM) to authenticate three medicinal Acanthaceae species (*Acanthus ebracteatus*, *Andrographis paniculata* and *Rhinacanthus nasutus*) commonly used in Thailand. The *rbc*L barcode was selected for use in primers design for HRM analysis to produce standard melting profiles of the selected species. Melting data from the HRM assay using the designed *rbc*L primers showed that the three chosen species could be distinguished from each other. HRM curves of all fifteen test samples indicated that three of tested products did not contain the indicated species. Two closely related species (*A*. *paniculata* and *R*. *nasutus*), which have a high level of morphological similarity, were interchanged with one another in three tested products. Incorrect information on packaging and labels of the tested herbal products was the cause of the results shown here. Morphological similarity among the species of interest also hindered the collection process. The Bar-HRM method developed here proved useful in aiding in the identification and authentication of herbal species in processed samples. In the future, species authentication through Bar-HRM could be used to promote consumer trust, as well as raising the quality of herbal products.

Introduction

Herbal medicines

Natural products from plants have played a considerable role in the way of life of people around the world since ancient times. Plant products have been consumed as food and used as medicinal remedies. An enormous number of scientific reports highlight the benefits of using medicinal plants and herbs as an alternative to modern synthetic drugs [1–4]. It is clear that medicinal plants are a popular alternative to synthetic drugs. According to the World Health Organization [5], over 70% of the world's population in developing countries uses herbal products. The past decade has seen the rapid growth of the herbal supplement and remedies market in many countries. Global Industry Analysts Inc. [6] report that the sale of these products has increased each year since 2004, with annual sales reaching 5.6 billion US dollars in 2012. It is estimated that the annual global sales of herbal supplement and remedies will reach up to 107 billion US dollar by the year 2017. The sale of these products in the US has risen for nine consecutive years since 2004. The report also notes that the global demand for herbal medicines continues to increase despite the economic recession. In addition to the US and Europe, Asia-Pacific and Japan also make up important markets for the global herbal supplement trade.

Many medicinal plant products have now been commercialized throughout various markets, including via the internet. These products are commonly sold in processed or modified forms such as powders, dried material, tablets, capsules and tea bags, making it almost impossible to accurately identify the constituent species [7,8]. Because of this, consumer safety could be a concern. Misidentification of the constituent plants may lead to the inclusion of undesirable, unrelated species, with a potential health risk to the end users. Substitution of the product's ingredients either intentionally or inadvertently can have negative effect on both consumers and producers. Herbal products are often perceived to be safe due to their natural origin. However, counterfeited, substituted and adulterated products can put consumers in danger [9–11]. Recent advances in molecular techniques have made it possible to detect substitution and adulteration in medicinal products, even in processed forms [12,13].

Herbal products found in Thai local markets are commonly sold without packaging or labels. Wholesale quantities of plant powders may be stored in large unlabeled bags or boxes, and as a result those who buy local herbal products have a high risk of attaining counterfeited, substituted and/or adulterated products. Regardless of the sellers' intention, buyers will never know whether the herbal powder they are buying is made of the species they intended to buy. Because of these identification issues, any measures that may aide in the identification of local herbal products would be beneficial. Many species from the Acanthaceae family are considered in Thailand to have health benefits, and three species (*Acanthus ebracteatus* Vahl, *Andrographis paniculata* (Burm.f.) Nees, and *Rhinacanthus nasutus* (L.) Kurz) are now included on the Thai National List of Essentials Medicine (NLEM; Thailand). These three Acanthaceae species are commonly used in Thai household as remedies for various ailments and thus are regularly sold at local markets. There is a need to find an approach that could help with the quality control of these herbal products to ensure both the satisfaction and safety of consumers.

DNA barcoding

DNA barcoding was developed about a decade ago, and relies on a short, standardized regions of the genome to identify plant and animal species [14,15]. The mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) gene was first chosen to be amplified and used to classify and identify butterfly species in the order Lepidoptera [16]. The results from this study show that butterfly species could be identified with 100% accuracy by using this short DNA region.

Since then, the rapid development of the method, along with an increased frequency of DNA barcode use in many fields that require species-level determination of organisms including animals, plants and microorganisms has proven the popularity of molecular barcoding [17–20]. The use of COI contributed to the discovery of a significant number of new animal species, including fish, birds, mammals, marine organisms and insects. More than 50,000 (30%) of the butterfly species in the order Lepidoptera have been investigated using DNA barcoding [16,21–23].

Although the use of DNA barcoding in animals is relatively widespread, similar use of the technique in the plant kingdom has not caught on as quickly [24,25]. COI is not suitable for plant identification because the locus in plant mtDNA has a low mutation rate, which results in too little variation to sufficiently discriminate among plant species [26]. Instead, chloroplast DNA (cpDNA) is more suitable for DNA barcoding in plants [24]. Several DNA regions in the chloroplast genome have shown sufficient variation to be useful for plant species identification. The CBOL Plant Working Group studied 907 plant species using a variety of gene and non-gene regions in the cpDNA [25]. As a result, the group proposed the use of two regions, *rbc*L and *mat*K, as DNA barcodes in plants, as they exhibit a promising efficiency in plant species discrimination [25]. As an alternative to cpDNA markers, the nuclear DNA internal transcribed spacer (ITS) region was recently also suggested as a good choice for species discrimination in plants [27–29].

Although DNA barcoding is proven useful for species-level identification of plants, there are some limitations to the technique. It is costly and time-consuming, and not easy to apply routinely in developing countries due to financial constraints and limited availability of perishable chemicals and consumables. The need to develop and validate a method that is still reliable, but more economical and rapid than DNA barcoding is an ongoing challenge. Here we applied DNA barcoding with high resolution melting (Bar-HRM) analysis for species identification and authentication. The use of Bar-HRM for taxonomic identification and the detection of adulteration in food and agriculture products has been reported recently [30–32]. In this study we evaluate whether the technique is equally useful for species discrimination in constituents of herbal products.

Methods

Primers used for HRM analysis

Sequences of the plastid DNA region, *rbc*L of selected medicinal plants from the family Acanthaceae were extracted from GenBank (at the end of February 2014) using the key phrases "the name of locus" and "the name of species" in the annotations. Generally, sequences obtained from public databases, including GenBank, are of low quality with no known associated herbarium vouchers. For this reason, all of the sequences were subjected to critical evaluation and any low-quality sequences were removed. After processing, multiple alignments were made from the selected sequences using MEGA6 [33] and variable characters were calculated for the design of primers to be used for high resolution melting (HRM) analysis. Two main criteria were considered in order to obtain successful results in the HRM analysis: (i) the primer pair should generate a PCR product not exceeding 300 bp, (ii) the primer pairs should cover enough variable sites to enable discrimination among the tested species.

Plant materials and DNA isolation

Both fresh and dried samples were included in this study. Three commonly used Thai herb species (*Ac. ebracteatus*, *An. paniculata* and *R. nasutus*) were the main focus of the study. Fresh specimens of these species were collected from The Garden of Medicinal Plants at the Faculty

of Pharmacy, Chiang Mai. Dried plant tissues for DNA extraction were kindly provided by Queen Sirikit Botanic Garden (QSBG) from the following herbarium vouchers (*Ac. ebracteatus*: QSBG voucher no. 29333, *An. paniculata*: QSBG voucher no. 68296 and *R. nasutus* QSBG voucher no. 63282). The plant material was ground with liquid nitrogen, and 100 mg of fine powder was then used for DNA extraction with the Nucleospin Plant II kit (Macherey-Nagel, Germany) following the manufacturer's instruction. The DNA was stored at −20° C for further use.

High resolution melting (HRM) analysis

To determine the characteristic melting temperature (T_m) for each sample that could be used to distinguish among the three different medicinal plants, DNA amplification using real-time PCR and DNA was performed using the Eco Real-Time PCR system (Illumina, San Diego, USA). The reaction mixture for the real-time PCR and HRM analysis consisted of a total volume of 10 μl, containing 5 μl of 2× THUNDERBIRD SYBR qPCR Mix, 0.2 μl of 10 mM forward primer, 0.2 μl of 10 mM reverse primer, 1 μl of 25 ng DNA and 3.6 μl of ddH_2O. The primer pair was derived from the *rbc*L sequence data retrieved from an online database (Forward 5'-TAGACCTTTTTGAAGAAGGTTCTGT-3' and Reverse 5'- TGAGGCGGRCCTTG GAAAGTT-3'). SYBR fluorescence dye was used to monitor both the accumulation of the amplified product and the high-resolution melting process in order to derive the T_m value during PCR.

The thermocycling reactions (PCR) were conducted in a 48-well Helixis plate using an initial denaturing step of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 20 s. The fluorescent data were acquired at the end of each extension step during the PCR. Before HRM, the products were denatured at 95°C for 15 s, and then annealed at 50°C for 15 s to randomly form DNA duplexes. For the HRM experiments, fluorescence data were collected every 0.1°C. Eco software (version 4.0.7.0) was used to analyze the T_m. The negative derivative of the fluorescence (F) over temperature (T) (dF/dT) curve displays the T_m, and the normalized raw curve depicts the decreasing fluorescence vs. increasing temperature. To generate normalized melting curves and differential melting curves [34], pre- and post-melt normalization regions were set to define the temperature boundaries of the normalized and difference plots that were used. *An. paniculata* was set as the reference species.

Authenticating test of herbal products sold on Thai local markets

Fifteen local products were purchased for this study. All of the products were acquired in powder form, without labeling and/or proper packaging. According to the sellers, two of the products were comprised of *Ac. ebracteatus*, eight products were comprised of *An. paniculata* and the remaining five were comprised of *R. nasutus* (Table 1). Total DNA was extracted from each sample and then used in HRM analysis in order to identify the characteristic melting temperature (T_m).

Results

Data mining and primers used

The amplification of the *rbc*L locus from three medicinal plant species in this study was performed using specific primers corresponding to the *rbc*L barcode region. All *rbc*L sequences of Acanthaceae were extracted from GenBank and the variable characters, average distance, and average %GC content were calculated for all samples using MEGA6. In total, 248 sequences were retrieved, of which 235 sequences were deemed useful for further analysis (S1 Table). An

Table 1. Bar-HRM identifications of the tested products.

Product number	Point of purchase	Latitude, Longitude coordinates	Putative species	Bar-HRM species identification
1	Ratchawong Road, Muang Chiang Mai	18.791346, 98.998703	*A. ebracteatus*	*A. ebracteatus*
2	Saturday Night Market, Sankamphaeng, Chiang Mai	18.742670, 99.122488	*A. ebracteatus*	*A. ebracteatus*
3	Warorot Market, Muang, Chiang Mai	18.790688, 99.001001	*R. nasutus*	*R. nasutus*
4	Ratchawong Road, Muang Chiang Mai	18.791346, 98.998703	*R. nasutus*	*R. nasutus*
5	Nawarat Market, Muang, Chiang Mai	18.791159, 99.000165	*R. nasutus*	*R. nasutus*
6	Warorot Market, Muang, Chiang Mai	18.790718, 99.000979	*R. nasutus*	*A. paniculata**
7	Saturday Night Market, Sankamphaeng, Chiang Mai	18.742670, 99.122488	*R. nasutus*	*R. nasutus*
8	Ratchawong Road, Muang Chiang Mai	18.791346, 98.998703	*A. paniculata*	*A. paniculata*
9	Nawarat Market, Muang, Chiang Mai	18.791159, 99.000165	*A. paniculata*	*A. paniculata*
10	Tonlamyai market, Muang, Chiang Mai	18.790287, 99.001289	*A. paniculata*	*A. paniculata*
11	Warorot Market, Muang, Chiang Mai	18.790688, 99.001001	*A. paniculata*	*R. nasutus**
12	Thapae Road, Muang, Chiang Mai	18.788176, 98.996893	*A. paniculata*	*A. paniculata*
13	Sunday Night Market Walking Street, Tha Pae Gate, Chiang Mai	18.787761, 98.993224	*A. paniculata*	*A. paniculata*
14	Saturday Night Market Walking Street, Wua Lai Road, Chiang Mai	18.778231, 98.984167	*A. paniculata*	*R. nasutus**
15	Saturday Night Market, Sankamphaeng, Chiang Mai	18.742670, 99.122488	*A. paniculata*	*A. paniculata*

*Species found to be different from the species indicated by the seller

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alignment of all useful sequences was made, and a 136 nucleotide base-pair fragment was analyzed after its amplification with newly developed forward and reverse primers. Thirty-six variable sites (26.5%) and 41.2% GC content were observed within the fragment (Fig 1A). The average distance was calculated for each sequence and plotted using Gephi [35] (Fig 1B). Fig 1B shows that all sequences from samples of the same genus cluster together. The majority of *rbc*L sequences are from *Justicia*. Commonly used Thai herbs belong predominantly to *Andrographis*, *Acanthus* and *Thunbergia*, though sequences from these genera are limited in our reference dataset. Among the total of 235 analyzed sequences, we found only one mismatched nucleotide position on the reverse primer site, and a perfect match among all samples for the forward primer. Thus, these newly developed primers, which were designed based on the sequences extracted from GenBank, were predicted to perform well in HRM analyses with the medicinal plant species in question.

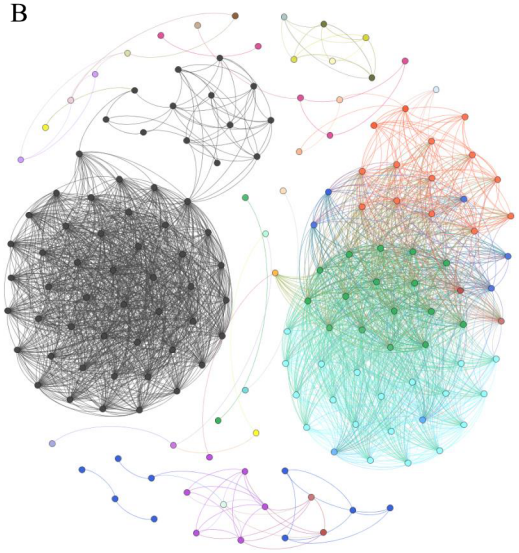
HRM analysis

The newly designed *rbc*L primer pair yielded amplicons of the expected size, approximately 136 base-pairs long. Eight variable sites were observed within the fragment of the three tested herb species (Fig 2A). The amplicons were analyzed using HRM to determine the T_m. Fig 2B and 2C present the analysis by means of conventional derivative plots, which show that the T_m value for the *rbc*L fragment from each species was represented by a peak. The melt curve is generated by slowly melting the DNA of tested plant species through a range of temperatures in the presence of a dsDNA binding dye. The melting temperature peaks of the tested plant species are calculated as T_m. HRM analysis with this primer pair proved to be a powerful tool for the identification of the three closely related Acanthaceae species (*Ac. ebracteatus*, *An. paniculata* and *R. nasutus*). The individual melting curves were reproducibly achieved from each of the three different species in triplicate analyses. Similar melting curves were achieved from the same species regardless of whether the DNA template was extracted from fresh or dried tissue

A

Base position	23	29	32	38	44	45	49	50	51	53	58	59	62	65	68	71	74	75
Nucleotide found	C	T	C	T	C	A	T	A	G	A	T	A	T	A	C	A	C	C
	T	C	T	C	G	T	A	G	C	G	G	G	G	G	T	T	A	T
Base position	**80**	**84**	**86**	**88**	**89**	**98**	**99**	**101**	**103**	**110**	**111**	**112**	**113**	**120**	**121**	**122**	**131**	**134**
Nucleotide found	T	C	A	G	T	T	C	G	G	T	C	C	T	G	T	T	C	A
	C	A	C	A	C	C	T	A	A	A	A	A	A	A	C	G	T	G
								T		C	G	T						

B



Genus	Number of sequence	Genus	Number of sequence
Justicia	63	Blepharis	1
Avicennia	24	Acanthopsis	1
Barleria	20	Crossandra	1
Ruellia	20	Phaulopsis	1
Aphelandra	20	Echinacanthus	1
Andrographis	12	Petalidium	1
Ruttya	7	Sanchezia	1
Thunbergia	7	Trichanthera	1
Eranthemum	4	Clarkeasia	1
Acanthus	4	Semnostachya	1
Nelsonia	4	Diceratotheca	1
Asystasia	3	Aechmanthera	1
Odontonema	3	Anisotes	1
Hypoestes	2	Pseuderanthemum	1
Monechma	2	Rungia	1
Peristrophe	2	Isoglossa	1
Rhinacanthus	2	Clinacanthus	1
Ruspolia	2	Mackaya	1
Baphicacanthus	2	Metarungia	1
Duvernoia	2	Dicliptera	1
Sclerochiton	2	Ecbolium	1
Hygrophila	2	Lepidagathis	1
Strobilanthes	2	Elytraria	1
Chaetacanthus	1		

Fig 1. Variable sites and Distance plot of the sequences retrieved from GenBank. A) Nucleotide variation in the 136 bp *rbc*L fragment of 235 aligned Acanthaceae species. B) Gelphi distance plot of the *rbc*L sequences belonging to 47 genera of Acanthaceae downloaded from GenBank.

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(Fig 2B and 2C). Thus, the method may also be applicable for processed herbal products that still contain tissue material with intact DNA.

Identification of herbal species in local products

Constituent species in herbal products bought from local markets in Thailand were investigated to assess the reliability of information regarding their ingredients, as the herbal products are often sold without proper packaging or labeling. Fifteen herbal products were purchased from local producers. The HRM analysis using *rbc*L primers was then performed to identify the species in the products and to see whether they are consistent with the species indicated by the sellers. The results of the analysis reveal that two samples which sellers identified as *An. paniculata* were actually *R. nasutus* (products no. 11 and 14), while one which sellers identified as *R. nasutus* was actually *An. paniculata* (product no. 6) (Fig 3).

Discussion

Whether intentional or not, substitution of species is not something that should happen. It is not possible to tell whether a product is the indicated species based on visual inspection, as they are sold in powdered form. Moreover, other on-site methods of identifying the component species studied here may be ineffective as both species taste similar (bitter) and lack a distinctive smell. In fact, when the morphology of these two herbal species is examined it becomes evident that they are relatively similar as it may be hard for a non-expert to distinguish or identify them. The Bar-HRM method developed here seems to be an effective way to accurately identify

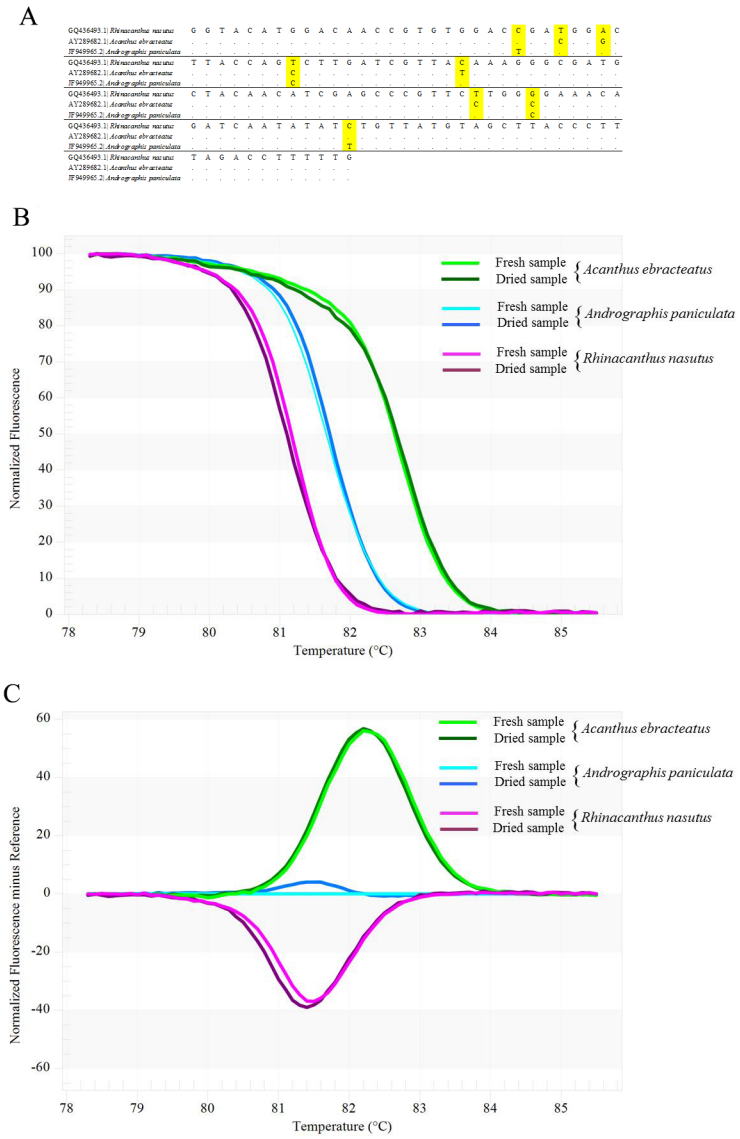

Fig 2. *rbc*L nucleotide alignment and melting profiles of the three Acanthaceae species. A) Variable sites detected in *rbc*L sequences of the three closely related species, *Acanthus ebracteatus*, *Andrographis paniculata* and *Rhinacanthus nasatus*. B) Normalized fluorescence plot of barcoding coupled high resolution melting (Bar-HRM). C) Reference-corrected normalized fluorescence plot of barcoding coupled high resolution melting (Bar-HRM).

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species in herbal products. Although twelve out of fifteen (80%) products tested were identified as the same species as indicated by the seller (Fig 3), a higher sample number as well as the inclusion of samples from more markets could lead to lower figures than 80%. It is not surprising that the misidentification of species in local products is reported here. The two main issues involved in this misidentification are that i) there is no reliable approach for controlling or authenticating raw materials collected by local producers and ii) there is no regulation on proper packaging or labeling of herbal products sold in Thai markets.

Detecting substitution and adulteration in herbal medicines using molecular techniques has been done before and proven to be a successful method [8,12,13]. For instance, one study found that a product labeled as black cohosh (*Actaea racemosa* L.), one of the top ten most

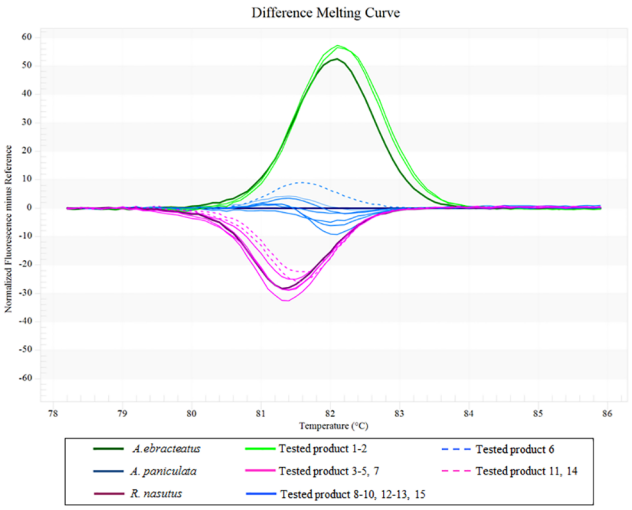

Fig 3. Representative profiles of the melting curves (difference plot curves). Bar-HRM difference plot curves obtained using the *rbc*L primer pair to identify 15 local herbal products. Three tested products (6, 11 and 14) were found to be different species from the species indicated by the vendor.

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popular herbal supplements sold in the US, actually contained three related Asian *Actaea* species that can be toxic to humans [36]. Studies in Morocco of the medicinal herbs trade found widespread substitutions of products sold on the markets [8,11,37]. It also showed that identifications made by herbalists selling retail herbal medicines were often inaccurate [37]. Another recent study of herbal medicines in North America that included 44 herbal products showed that almost half of the products contained species that were not listed among the main ingredients on the label [13]. Surprisingly, another third of the products tested contained none of the ingredients indicated on the label [13]. Several other studies have raised similar concerns from their findings. A study of 146 commercial herbal teas based on either *rbc*L or *mat*K barcodes revealed that 35% of the samples were contaminated with ingredients that were not on the labels [38]. A study of Korean and American ginseng using *rbc*L, *mat*K and ITS barcodes found that 50% of the ginseng products examined in the study contained American ginseng (*Panax quinquefolius* L.) instead of Asian ginseng (*Panax ginseng* C.A.Mey.) [39].

These examples have all used standard Sanger barcoding or metabarcoding approaches, but the use of Bar-HRM to study herbal medicine substitution has not been reported before [40]. Bar-HRM of botanical products has been successfully used for the authentication of an EU Protected Designation of Origin product made from *Lathyrus clymenum* [41], for olive oil and adulterants [42], for species distinction in Mediterranean pines [31], for detection of allergenic hazelnut contamination [32], for processed bean crops [30,43,44]. Bar-HRM is quickly gaining popularity in application due to its low direct and indirect costs and high accuracy of detection.

Conclusion

Pharmacovigilance of herbal medicines relies on product label information of ingredients and the adherence to good manufacturing practices along the commercialization chain. Several studies have shown that substitution of plant species occurs in herbal medicines, and this in turn poses a challenge to herbal pharmacovigilance as adverse reactions might be due to substituted ingredients. Bar-HRM analysis has been proven to be a fast and reliable technique for the authentication of herbal products. Here, we describe the development of a Bar-HRM method that can be used to test for the adulteration of commonly used herbal products. The tested

products were traded as processed powder, which impedes conventional identification. Because of this processing it is almost impossible to identify which herbal species are present in products using morphological characters. The DNA extracted from all products tested yielded a specific amplification product with the designed *rbc*L Bar-HRM primers. The normalised HRM curves for the amplicons, from the three species (*An. paniculata*, *Ac. ebracteatus* and *R. nasutus*) and 15 herbal products, based on HRM analysis with barcode marker *rbc*L were easily distinguished, and 12 of the 15 tested samples were successfully assigned to the species indicated by the seller. However, three products were found to contain plant species that were different from the species indicated by the seller. Therefore, the developed method could be easily used for rapid and low-cost authentication of herbal products.

Supporting Information

S1 Table. Acanthaceae sequences of rbcL were retrieved from GenBank (NCBI) for each of the genus with accession number.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MO PM. Performed the experiments: MO. Analyzed the data: MO. Contributed reagents/materials/analysis tools: MO. Wrote the paper: MO HdB.

References

1. De Smet PA (1997) The role of plant-derived drugs and herbal medicines in healthcare. Drugs 54: 801–840. PMID: 9421691
2. Calixto BJ (2000) Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res 33: 2.
3. Rates SMK (2001) Plants as source of drugs. Toxicon 39: 603–613. PMID: 11072038
4. Gurib-Fakim A (2006) Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol Aspects Med 27: 1–93. PMID: 16105678
5. World Health Organization (2003) Traditional Medicine. Factsheet No. 134. Available: http://www.who.int/mediacentre/factsheets/fs134/en/.
6. GIA (2013) Herbal Supplements and Remedies—A Global Strategic Business Report. San Jose, USA: Global Industry Analysts Inc.
7. Veldman S, Otieno J, Gravendeel B, Andel T van, Boer H de (2014) Conservation of Endangered Wild Harvested Medicinal Plants: Use of DNA Barcoding. Nov Plant Bioresour Appl Food Med Cosmet: 81–88.
8. Kool A, de Boer HJ, Krüger Å, Rydberg A, Abbad A, Björk L, et al. (2012) Molecular identification of commercialized medicinal plants in Southern Morocco. PLoS ONE 7: e39459. doi: 10.1371/journal.pone.0039459 PMID: 22761800
9. Ize-Ludlow D, Ragone S, Bruck IS, Bernstein JN, Duchowny M, Peña BM (2004) Neurotoxicities in infants seen with the consumption of star anise tea. Pediatrics 114: e653. doi: 10.1542/peds.2004-0058 PMID: 15492355
10. Skalli S, Alaoui I, Pineau A, Zaid A, Soulaymani R (2002) L'intoxication par le chardon à glu (*Atractylis gummifera* L.): à propos d'un cas clinique. Bull Soc Pathol Exot 95: 284–286. PMID: 12596380
11. Ouarghidi A, Powell B, Martin GJ, de Boer HJ, Abbad A (2012) Species substitution in medicinal roots and possible implications for toxicity in Morocco. Econ Bot 66: 370–382.

12. Coghlan M, Haile J, Houston J, Murray D, White N, Moolhuijzen P, et al. (2012) Deep sequencing of plant and animal DNA contained within traditional chinese medicines reveals legality issues and health safety concerns. PLoS Genet 8: e1002657. doi: 10.1371/journal.pgen.1002657 PMID: 22511890
13. Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S (2013) DNA barcoding detects contamination and substitution in North American herbal products. BMC Med 11: 222. doi: 10.1186/1741-7015-11-222 PMID: 24120035
14. Hebert PDN, Cywinska A, Ball S, de Waard J (2003) Biological identifications through DNA barcodes. Proc R Soc B 270: 313–322. PMID: 12614582
15. Hebert PD, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Syst Biol 54: 852–859. PMID: 16243770
16. Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci U S A 101: 14812–14817. PMID: 15465915
17. Chase MW, Fay MF (2009) Barcoding of plants and fungi. Science 325: 682. doi: 10.1126/science.1176906 PMID: 19644072
18. Crawford AJ, Cruz C, Griffith E, Ross H, Ibáñez R, Lips KR, et al. (2012) DNA barcoding applied to ex situ tropical amphibian conservation programme reveals cryptic diversity in captive populations. Mol Ecol Resour.
19. Fazekas AJ, Kesanakurti PR, Burgess KS, Percy DM, Graham SW, Barrett SC, et al. (2009) Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? Mol Ecol Resour 9: 130–139. doi: 10.1111/j.1755-0998.2009.02652.x PMID: 21564972
20. Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Biol 2: e312. PMID: 15455034
21. Silva-Brandão KL, Lyra ML, Freitas AV (2009) Barcoding Lepidoptera: current situation and perspectives on the usefulness of a contentious technique. Neotrop Entomol 38: 441–451. PMID: 19768260
22. Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PD (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci U S A 103: 968–971. PMID: 16418261
23. Burns JM, Janzen DH, Hajibabaei M, Hallwachs W, Hebert PDN (2008) DNA barcodes and cryptic species of skipper butterflies in the genus *Perichares* in Area de Conservacion Guanacaste, Costa Rica. Proc Natl Acad Sci 105: 6350–6355. doi: 10.1073/pnas.0712181105 PMID: 18436645
24. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci 102: 8369–8374. PMID: 15928076
25. CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci 106: 12794–12797. doi: 10.1073/pnas.0905845106 PMID: 19666622
26. Cho Y, Mower JP, Qiu Y, Palmer JD (2004) Mitochondrial substitution rates are extraordinarily elevated and variable in a genus of flowering plants. PNAS 101: 17741–17746. PMID: 15598738
27. Li DZ, Gao LM, Li HT, Wang H, Ge XJ, Liu JQ, et al. (2011) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci 108: 19641–19646. doi: 10.1073/pnas.1104551108 PMID: 22100737
28. Chen S, Yao H, Han J, Liu C, Song J, Shi L, et al. (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE 5: 1–8. doi: 10.1371/journal.pone.0008613
29. Hollingsworth PM (2011) Refining the DNA barcode for land plants. Proc Natl Acad Sci U S A 108: 19451–19452. doi: 10.1073/pnas.1116812108 PMID: 22109553
30. Madesis P, Ganopoulos I, Anagnostis A, Tsaftaris A (2012) The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (Leguminosae) without prior DNA purification. Food Control 25: 576–582.
31. Ganopoulos I, Aravanopoulos F, Madesis P, Pasentsis K, Bosmali I, Ouzounis C, et al. (2013) Taxonomic identification of Mediterranean pines and their hybrids based on the high resolution melting (HRM) and trnL approaches: from cytoplasmic inheritance to timber tracing. PloS One 8: e60945. doi: 10.1371/journal.pone.0060945 PMID: 23577179
32. Madesis P, Ganopoulos I, Bosmali I, Tsaftaris A (2013) Barcode High Resolution Melting analysis for forensic uses in nuts: A case study on allergenic hazelnuts (*Corylus avellana*). Food Res Int 50: 351–360.
33. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353
34. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49: 853–860. PMID: 12765979

35. Bastian M, Heymann S, Jacomy M, others (2009) Gephi: an open source software for exploring and manipulating networks. ICWSM 8: 361–362.

36. Baker DA (2012) DNA barcode identification of black cohosh herbal dietary supplements. J AOAC Int 95: 1023–1034. PMID: 22970567

37. De Boer HJ, Ouarghidi A, Martin G, Abbad A, Kool A (2014) DNA Barcoding Reveals Limited Accuracy of Identifications Based on Folk Taxonomy. PLOS ONE 9: e84291. doi: 10.1371/journal.pone.0084291 PMID: 24416210

38. Stoeckle MY, Gamble CC, Kirpekar R, Young G, Ahmed S, Little DP (2011) Commercial teas highlight plant DNA barcode identification successes and obstacles. Sci Rep 1: 1–7. doi: 10.1038/srep00042 PMID: 22355520

39. Wallace LJ, Boilard SM, Eagle SH, Spall JL, Shokralla S, Hajibabaei M (2012) DNA barcodes for everyday life: Routine authentication of Natural Health Products. Food Res Int 49: 446–452.

40. Madesis P, Ganopoulos I, Sakaridis I, Argiriou A, Tsaftaris A (2014) Advances of DNA-based methods for tracing the botanical origin of food products. Food Res Int 60: 163–172.

41. Ganopoulos I, Madesis P, Darzentas N, Argiriou A, Tsaftaris A (2012) Barcode High Resolution Melting (Bar-HRM) analysis for detection and quantification of PDO "Fava Santorinis" (*Lathyrus clymenum*) adulterants. Food Chem 133: 505–512. doi: 10.1016/j.foodchem.2012.01.015 PMID: 25683426

42. Ganopoulos I, Bazakos C, Madesis P, Kalaitzis P, Tsaftaris A (2013) Barcode DNA high-resolution melting (Bar-HRM) analysis as a novel close-tubed and accurate tool for olive oil forensic use. J Sci Food Agric 93: 2281–2286. doi: 10.1002/jsfa.6040 PMID: 23400707

43. Bosmali I, Ganopoulos I, Madesis P, Tsaftaris A (2012) Microsatellite and DNA-barcode regions typing combined with High Resolution Melting (HRM) analysis for food forensic uses: A case study on lentils (*Lens culinaris*). Food Res Int 46: 141–147.

44. Ganopoulos I, Madesis P, Tsaftaris A (2012) Universal ITS2 barcoding DNA region coupled with high-resolution melting (HRM) analysis for seed authentication and adulteration testing in leguminous forage and pasture species. Plant Mol Biol Report 30: 1322–1328.

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Fast and reliable detection of toxic *Crotalaria spectabilis* Roth. in *Thunbergia laurifolia* Lindl. herbal products using DNA barcoding coupled with HRM analysis

Sahachat Singtonat and Maslin Osathanunkul*

Abstract

Background: Nowadays, medicinal plants are used as a popular alternative to synthetic drugs. Many medicinal plant products have now been commercialized throughout various markets. These products are commonly sold in processed or modified forms such as powders, dried material and capsules, making it almost impossible to accurately identify the constituent species. The herbal plant known as 'Rang Chuet' in Thai has been widely used as remedies for various ailments. However, two medicinal plants species, *Thunbergia laurifolia* and *Crotalaria spectabilis* share this name. Duo to the similarity in nomenclature, the commercial products labeled as 'Rang Chuet' could be any of them. Recently, the evidence of hepatotoxic effects linked to use of *C. spectabilis* were reported and is now seriously concern. There is a need to find an approach that could help with species identification of these herbal products to ensure the safety and efficacy of the herbal drug.

Methods: Here DNA barcoding was used in combination with High Resolution Melting analysis (Bar-HRM) to authenticate *T. laurifolia* species. Four DNA barcodes including *matK*, *rbcL*, *rpoC* and *trnL* were selected for use in primers design for HRM analysis to produce standard melting profiles of the selected species. Commercial products labeled as 'Rang Chuet' were purchased from Thai markets and authentication by HRM analyses.

Results: Melting data from the HRM assay using the designed primers showed that the two 'Rang Chuet' species could easily be distinguished from each other. The melting profiles of the all four region amplicons of each species are clearly separated in all three replicates. The method was then applied to authenticate products in powdered form. HRM curves of all ten test samples indicated that three of the tested products did not only contain the *T. laurifolia* species.

Conclusion: The herbal drugs derived from different plants must be distinguished from each other even they share the same vernacular name. The Bar-HRM method developed here proved useful in the identification and authentication of herbal species in processed samples. In the future, species authentication through Bar-HRM could be used to promote consumer trust, as well as raising the quality of herbal products.

Background

Herbal medicines

Natural products from plants have played a considerable role in the way of life of people around the world since ancient times. Plant products have been consumed as food and used as medicinal remedies. An enormous number of scientific reports highlight the benefits of using medicinal plants and herbs as an alternative to modern synthetic drugs [1]. It is clear that medicinal plants are a popular alternative to synthetic drugs. According to the World Health Organization [2], over 70% of the world's population in developing countries uses herbal products. Many medicinal plant products have now been commercialized. These products are commonly sold in processed or modified forms such as powders, dried material, tablets, capsules and tea bags, making it almost impossible to accurately identify the constituent species [3–5]. Because of this, consumer

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safety could be a concern. Misidentification of the constituent plants may lead to the inclusion of undesirable, unrelated species, with a potential health risk to the end users. Substitution of the product's ingredients either intentionally or inadvertently can have negative effect on both consumers and producers. Because of these identification issues, any measures that may aide in the identification of herbal products would be beneficial. Many species from the Acanthaceae family are considered in Thailand to have health benefits, several species (*Acanthus ebracteatus*, *Andrographis paniculata*, *Rhinacanthus nasutus* and *Thunbergia laurifolia*) are now included on the Thai National List of Essentials Medicine (NLEM; Thailand). These Acanthaceae species are commonly used in Thai household as remedies for various ailments and thus are regularly sold on the markets throughout Thailand. There is a need to find an approach that could help with the quality control of these herbal products to ensure both the satisfaction and safety of consumers.

Thunbergia laurifolia is one of common Thai medicinal plant with various used such as antipyretic, detoxification against insecticides, alcoholic and metallic poisons. *T. laurifolia* commonly known in Thai as 'Rang Chuet' has been used in Thailand as a natural remedy for decades. Commercial products of 'Rang Chuet' in tea, capsule, and powder forms in herbal markets are claimed to have beneficial effects on human health. However, confusion has arisen because of the similarity in the vernacular names of the plants. In Thailand, there are at least three species are being called 'Rang Chuet', one of these is *Crotalaria spectabilis*. There are several documentations reported that seeds and leaves of *C. spectabilis* contain pyrrolizidine alkaloids which causes Hepatotoxicity in humans and mammals [6–10]. Due to the fact that, Rang Chuet was immensely sold in Thailand local markets and used as household remedy in form of processed products. Therefore it is almost impossible for consumer to know exactly which 'Rang Chuet' products they are buying so this could be a real big issue and is now seriously concern.

Molecular species identification

Recently, many works have been focused on authentication or detection of species substitution of herbal products. Molecular techniques like RFLP and RAPD were developed for Rang Chuet identification [4, 5]. Both RFLP and RAPD technique showed a potential in discrimination of the two Rang Chuet (*T. laurifolia* and *C. spectabilis*) species but the disadvantage of RFLP is not only relatively time consuming but the RFLP also requires a large amount of sample, although RAPD technique is fast but many [11–14] show unstable results and sometimes unreproducible.

In the past decade, DNA barcoding (short DNA sequence) is proved to be useful for identifying and categorizing species [15]. To date, several Rang Chuet barcodes from various plastid genome regions including *matK*, *rpl*16, *rps*16, *trnL* and *rbcL* were produced [5, 14]. Although DNA barcoding is proven useful for species-level identification of plants [16, 17], there are some limitations to the technique. It is costly and time-consuming, and not easy to apply routinely in developing countries due to financial constraints and limited availability of perishable chemicals and consumables. This leads us in searching for new fast, reliable, less time consuming and inexpensive method for species identification and authenticating of medicinal plants. Here we applied DNA barcoding with high resolution melting (Bar-HRM) analysis for species identification and authentication of 'Rang Chuet' products sold on Thai markets. The use of Bar-HRM for taxonomic identification and the detection of adulteration in food and agriculture products has been reported recently [18–20]. In this study we evaluate whether the technique is equally useful for species discrimination in constituents of Thai folk medicinal plant.

Materials and methods

Primers used for HRM analysis

Sequences of the plastid DNA regions, *matK, rbcL*, *rpoC* and *trnL* of selected medicinal plants from the family Acanthaceae (*Thunbergia* spp.) and Fabaceae (*Crotalaria* spp.) were extracted from GenBank (at the end of September 2013) using the key phrases "the name of locus" and "the name of species" in the annotations. Generally, sequences obtained from public databases, including GenBank, are of low quality with no known associated herbarium vouchers. For this reason, all of the sequences were subjected to critical evaluation and any low-quality sequences were removed. After processing, multiple alignments were made from the selected sequences using MEGA6 [21] and variable characters were calculated for the design of primers to be used for high resolution melting (HRM) analysis. Two main criteria were considered in order to obtain successful results in the HRM analysis: (i) the primer pair should generate a PCR product not exceeding 300 bp, (ii) the primer pairs should cover enough variable sites to enable discrimination among the tested species and any other variation site from sequence of conspecifics.

Plant samples and DNA isolation

Both fresh and dried samples were included in this study (Table 1). Two 'Rang Chuet' herb species (*T. laurifolia* and *C. spectabilis*) were the main focus of the study. Fresh specimens of these species were collected from areas in Chiang Mai province, Thailand. Dried plant tissues for DNA extraction were kindly provided by Queen

Table 1 Plants species and commercial products included in this study

Species/Type	Abbreviation	Source	Sample type
Thunbergia laurifolia	T1	Materia Medica garden	Fresh
		Faculty of Pharmacy, Chiang Mai University	
Thunbergia laurifolia	T2	Department of Biology	Fresh
		Faculty of Science, Chiang Mai University	
Thunbergia laurifolia	T3	Queen Sirikit Botanical Garden	Dry
		Mae Rim, Chiang Mai (voucher number 46323)	
Thunbergia laurifolia	T4	Queen Sirikit Botanical Garden	Dry
		Mae Rim, Chiang Mai (voucher number 59427)	
Crotalaria spectabilis	C1	Materia Medica garden	Fresh
		Faculty of Pharmacy, Chiang Mai University	
Crotalaria spectabilis	C2	Materia Medica garden	Fresh
		Faculty of Pharmacy, Chiang Mai University	
Crotalaria spectabilis	C3	CMU Biology Garden	Fresh
		Faculty of Science, Chiang Mai University	
Commercial CN-C	COM1	Chiang Mai	Capsule
Commercial TT-C	COM2	Chiang Mai	Capsule
Commercial HBO-C	COM3	Chiang Mai	Capsule
Commercial HBO-T	COM4	Chiang Mai	Tea bag
Commercial APB-P	COM5	Lamphun	Powder
Commercial NK-L	COM6	Lamphun	Dried leaf
Commercial GT-L	COM7	Pa Yao	Dried leaf
Commercial OTOP-T	COM8	Pa Yao	Tea bag
Commercial RTN-S1	COM9	Pa Yao	Dried bark
Commercial RTN-S2	COM10	Pa Yao	Dried bark

Sirikit Botanic Garden (QSBG). The plant material was ground with liquid nitrogen, and then used for DNA extraction with the Nucleospin Plant® II kit (Macherey-Nagel, Germany) following the manufacturer's instruction. DNA concentrations of all samples were equally adjusted (20 ng/μL). The DNA was stored at−20° C for further use.

Real-time PCR amplification and high resolution melting (HRM) analysis

To determine the characteristic melting temperature (T_m) for each sample that could be used to distinguish the two different 'Rang Chuet' medicinal plants, PCR amplification, DNA melting, and end point fluorescence level acquiring PCR amplifications were performed in a total volume of 20 μL on an Eco™ Real-Time PCR system (Illumina®, San Diego, USA). The reaction mixture contained 10 ng genomic DNA, 10 μL of MeltDoctor™ HRM Master Mix (Applied Biosystems, California, USA), 0.2 μL of 10 mM forward and reverse primers. The four pairs of candidate barcoding primers nucleotide composition are shown in Table 2. The real-time PCR reaction conditions are as following; an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 20 s. Subsequently, the PCR amplicons were denatured for HRM at 95 °C for 15 s, and then annealed at 50 °C for 15 s to form random DNA duplexes. Melting curves were generated after the last extension step. The temperature was increased from 60 to 95 °C at 0.1 °C/s. The melting curves were analyzed with the Eco™ software (version 4.0.7.0). After obtaining the suitable primers for the HRM in order to test the sensitivity of the developed

Table 2 Four primers used for HRM analysis and identification

Primer name	Nucleotide sequence (5′ to 3′)	T_a (°C)	Expected size (bp)
matK _F	CTTCTTATTTACGATTAACATCTTCT	57	160
matK _R	TTTCCTTGATATCGAACATAATG		
rbcL_F	GGTACATGGACAACTGTGTGGA	57	150
rbcL _R	ACAGAACCTTCTTCAAAAAGGTCTA		
rpoC_F	CCSATTGTATGGGAAATACTT	57	170
rpoC _R	CTTACAAACTAATGGATGTAA		
trnL_F	GAATCGACCGTTCAAGTATCC	57	150
trnL _R	TATAGGAAACCCATATTTGATCCAATC		

method, real-time PCR and barcoding with HRM were carried out on standard samples, prepared by mixing fine powder of *T. laurifolia* with *C. spectabilis* in different proportions of 1, 3, 6, 12, 25, and 50 %. Real-time PCR amplification was performed as described earlier.

Authenticating test of herbal products sold on Thai local markets

Ten herbal products labeled as 'Rang Chuet' were purchased for this study. All of the products were acquired in processed forms (Table 1). Total DNA was extracted from each sample and then used in HRM analysis in order to identify the characteristic melting temperature (T_m).

Results and discussion

Data mining and primers used

The amplification of the four selected locus from two 'Rang Chuet' medicinal plant species (*T. laurifolia* and *C. spectabilis*) was performed using specific primers corresponding to the *matK*, *rbcL*, *rpoC* and *trnL* barcode region. All sequences of *Thunbergia* spp. were extracted from GenBank and the variable characters and average %GC content was calculated for all samples (Table 3). Data was present for most markers of the target species, except for *rpoC*. The total number of sequences retrieved for the respective markers were: *matK* 11 (7 species); *rbcL* 6 (6 species), *trnL* 27 (20 species). The absence of *rpoC* sequences for the target species was resolved by selecting random *rpoC* sequences from GenBank, which is supported by the high universality of *rpoC* [17, 22]. Two sequences of *C. spectabilis* were retrieved (*matK* and *trnL*).

For *matK* 5, *rbcL* 6, *rpoC* 5 and *trnL* 19 sequences were deemed useful for further analysis (Table 4). An alignment of all useful sequences was made, and the primers flanking regions of each marker ranging from 150 to 170 bp were analyzed (Table 2). Reed and Wittwer [23] found that suitable length for HRM analysis should be 300 bp or less for optimal results.

Both the sequence length and the nucleotide variation within sequences influence the dissociation energy of the base pairs and result in different T_m values. The *matK* amplicon sequences were observed to have higher nucleotide variation than the amplicons of the other regions, at 30.67%. The relative nucleotide variation within amplicons was found to be as follows: *matK* > *rpoC* > *trnL* > *rbcL* (Table 3). The forward and reverse *matK* primers matched the consensus sequence of the target species at the binding sites in only 15 out of 26 sites (57.69%) and 19 out 23 of sites (82.61%), respectively (Table 3). High universality at the initial bases of the primer site is crucial for primer annealing and subsequent elongation initiation by the DNA polymerase. The *matK* locus is one of the most variable plastid coding regions and has high interspecific divergence and good discriminatory power. However, it can be difficult to amplify with the standard barcoding primers due to high substitution rates at the primer sites [24, 25]. The *rbcL, rpoC* and *trnL* primer pairs were expected to be a suitable primer for HRM analysis for discrimination between the tested plant species. These primers were nearly identical in base similarity to the mined consensus sequence (Table 3).

The average %GC content of amplicons was calculated in order to predict variation in melting curves for the different markers. *trnL* had the lowest average %GC content, with 34.50%, followed by *matK*, *rpoC* and *rbcL*, with 35.20, 41.85, 44.26 and 46.60% respectively (Table 3).

Finding suitable primer pairs for discrimination between *T. laurifolia* and *C. spectabilis*

The four primers sets were used for the amplification of DNA-fragments from all seven samples (two 'Rang Chuet' species), and the amplicons were analyzed using HRM to define T_m. (Table 5). The expected length of amplified products from *matK*, *rbcL*, *rpoC*, and *trnL* are 160 bp, 150 bp, 170 bp, and 150 bp, respectively. The melting profiles of all amplicons are illustrated in Fig. 1a-1d. The analysis is presented by means of conventional derivative plots, which show that the T_m value of each species is represented by a peak. The samples of the two different species could be easily distinguished using HRM analysis with all four primer pairs. The melting profiles of seven samples of the two 'Rang Chuet' species (*T. laurifolia* and *C. spectabilis*) can be divided into two groups. All *T. laurifolia* samples (T1-T4) are grouped together and the other group contains all *C. spectabilis* samples (C1-C3). Although

Table 3 Characteristics of sequences and primers for high resolution melting analysis

Regions	*matK*	*rbcL*	*rpoC*	*trnL*
Available species	5	6	5	19
Variable characters (%)	30.67	10.07	10.19	8.90
Conserved forward primer/total (%)	15/26 (57.69)	21/22 (95.45)	18/21 (85.71)	19/21 (90.48)
Conserved reverse primer/total (%)	19/23 (82.61)	25/25 (100)	19/21 (90.48)	24/27 (88.89)
Average %GC content	35.20	46.60	44.26	34.50

Table 4 Sequences of four plastid regions (*matK, rbcL, rpoC* and *trnL*) were retrieved from GenBank (NCBI) for each of the species with accession number

Species	Regions			
	trnL	*matK*	*rbcL*	*rpoC*
Crotalaria spectabilis	HM208335	AB649973	-	-
Thunbergia affinis	AB817377	-	-	-
	EU315886			
Thunbergia alata	AF061820	HQ384512	HQ384878	-
	EU529130	AF531811		
	EU315887			
Thunbergia angulata	EU315888	-	-	-
Thunbergia arnhemica	EU315889	-	-	-
Thunbergia atriplicifolia	EU315890	-	-	-
Thunbergia battiscombei	EU315891	-	-	-
Thunbergia capensis	EU315892	AM234783	AM234783	-
Thunbergia coccinea	EU529131	HG004920	KF181493	-
Thunbergia convolvulifolia	EU315894	-	-	-
Thunbergia dregeana	EU315895	-	-	-
Thunbergia erecta	AF061821	AB649972	-	-
	JQ764614			
	EU529132			
	EU315896			
Thunbergia fragrans	U315897	-	-	-
Thunbergia galpinii	EU315898	-	-	-
Thunbergia grandiflora	EU315899	AB649971	JQ590086	-
		JQ586429		
		JQ586428		
		JQ586427		
Thunbergia gregoryi	EU315901	-	-	-
Thunbergia guerkeana	EU315901	-	-	-
Thunbergia kirkii	EU315902	-	-	-
Thunbergia laurifolia	-	AB649970	-	-
Thunbergia mysorensis	-	-	AY008828	-
Thunbergia petersiana	EU315904	-	-	-
Thunbergia pondoensis	EU315905	-	-	-
Thunbergia togoensis	EU315906	-	-	-
Thunbergia usambarica	-	-	L12596	-

the four primer pairs tested could be used to discriminate *T. laurifolia* from *C. spectabilis*, the *rpoC* region was chosen for further analysis as it would help in demonstrating that Bar-HRM could work well as a sequencing-free method for plant identification. In addition, the *rpoC* region was used as an analytical target in HRM analysis has been shown to be effective for the detection and quantification of *Lens culinaris* and *Lathyrus clymenum* adulterations [18, 22].

Quantitative detection of *T. laurifolia* adulterants with Bar-HRM analysis

Detecting limit of adulteration in *T. laurifolia* products using the developed method with *rpoC* primers was tested. Figure 2a shows the results of the validation method with *T. laurifolia* spiked with *C. spectabilis* in different proportions. These results depict the analysis for one experiment as all three experiments gave similar results thus showing very good reproducibility. The process of the *T. laurifolia* amplicon dissociation reveals the level of contamination resulting from adulteration as the presence of increasing quantity of *C. spectabilis* into the *T. laurifolia* DNA alters the shape and shifts proportionally the melting curve, compared to the curve of pure *T. laurifolia* DNA. By applying this approach, we were able to detect adulterations as low as 1 % (Fig. 2a).

Identification of herbal species in commercial products

Constituent species in herbal products bought from markets in Thailand were investigated to assess the reliability of information regarding their ingredients, as the herbal products are often sold in processed forms. Ten herbal products labeled as 'Rang Chuet' were purchased and examined (Table 1). The HRM analysis using *rpoC* primers was then performed to identify the species in the products.

The examination of the HRM difference curve of all tested samples using *T. laurifolia* curve as baseline revealed that seven out of ten samples (COM3-7 and 9–10) produce curves in which the same as *T. laurifolia*'s with a 90% confidence interval, suggesting that the products contain *T. laurifolia* (Fig. 2b). The melting curve of one tested sample (COM8) was found between *T. laurifolia* and *C. spectabilis* lines, it could be indicated that the commercial COM8 was probably be admixture of *T. laurifolia* and *C. spectabilis* with around 3% of the toxic *C. spectabilis* in the product as show in Fig. 2b. However, we cannot rule out the possibility of the COM8 may actually not be contaminated with the toxic *C. spectabilis* but other species. In addition, the results of the analysis also reveal that the two remaining samples (COM1 and COM2) were much likely not contain any of the two 'Rang Chuet' species but some other species instead (Fig. 2b). In order to find contaminated or substituted species in COM1 and COM2, DNA barcoding is one of the best solutions. As can be seen from Newmaster *et al* [26] work, DNA barcoding was performed to detect the adulteration and substitution of herbal drugs and found that herbal products sold on the markets were contaminated or substituted with alternative plant species that are not listed on the labels as they are replaced entirely by powdered rice, wheat and soybean. Thus, DNA sequencing of *rbcL* region was carried out to identify species in these two products. The blast result showed that COM1 and COM2 have a similarity in their

Table 5 The values of melting temperature (°C) with standard deviations gaining form high resolution melting (HRM) analysis using *matK*, *rbcL*, rpoC and *trnL* primers of *T. laurifolia* and *C. spectabilis* species

Species	Abbreviation	Tm (°C)			
		matK	*rbcL*	*rpoC*	*trnL*
T. laurifolia	T1	-	82.5 ± 0.07	80.2 ± 0.12	78.8 ± 0.07
T. laurifolia	T2	-	82.4 ± 0.14	80.1 ± 0.15	78.9 ± 0.07
T. laurifolia	T3	77.9 ± 0.35	82.3 ± 0.14	80.2 ± 0.00	78.8 ± 0.00
T. laurifolia	T4	-	82.4 ± 0.07	80.2 ± 0.07	78.8 ± 0.14
C. spectabilis	C1	75.6 ± 0.21	81.6 ± 0.00	80.9 ± 0.06	78.4 ± 0.14
C. spectabilis	C2	75.3 ± 0.07	81.6 ± 0.07	80.9 ± 0.06	-
C. spectabilis	C3	75.4 ± 0.21	81.5 ± 0.00	80.8 ± 0.00	78.2 ± 0.00

(-) No amplicons were generated

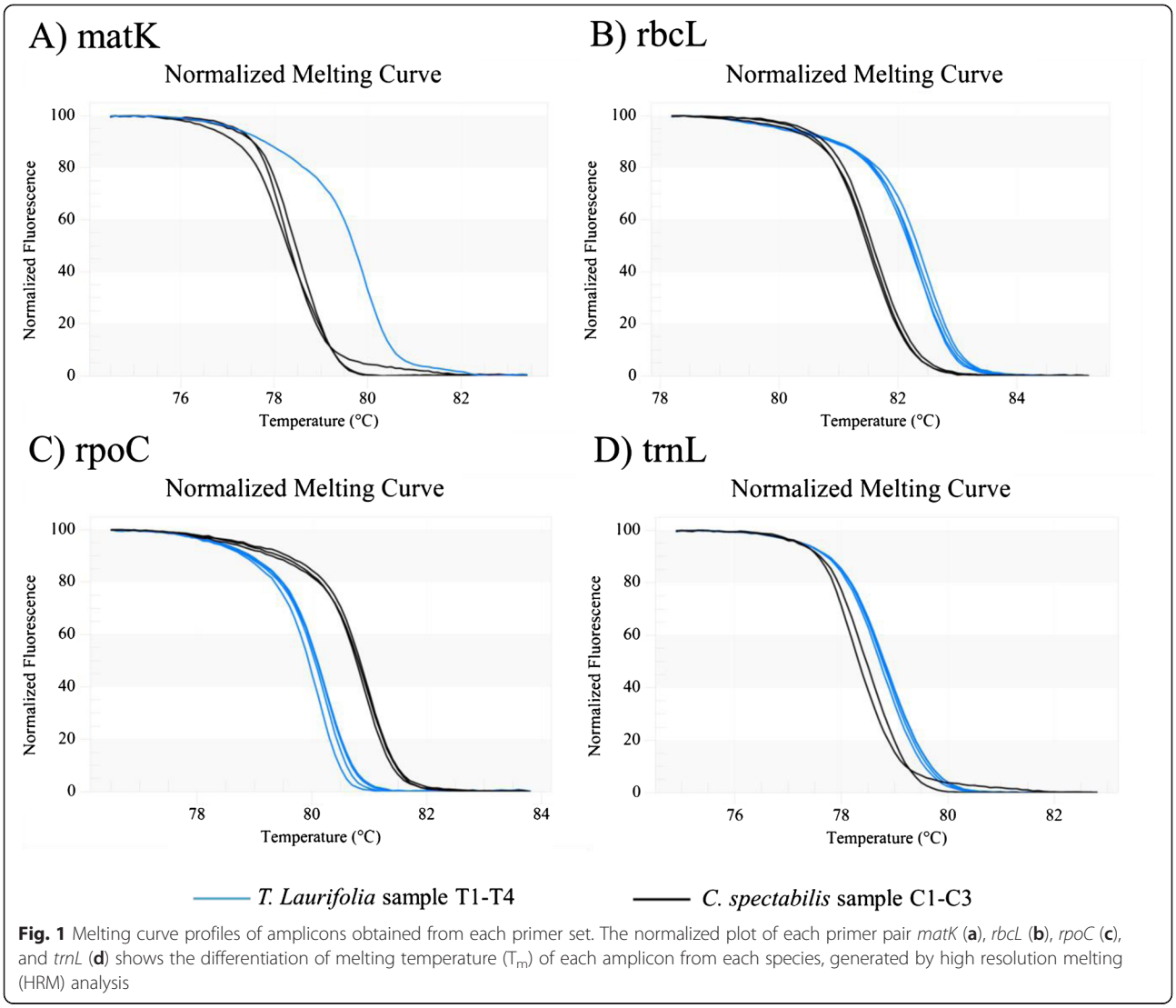

Fig. 1 Melting curve profiles of amplicons obtained from each primer set. The normalized plot of each primer pair *matK* (**a**), *rbcL* (**b**), *rpoC* (**c**), and *trnL* (**d**) shows the differentiation of melting temperature (T_m) of each amplicon from each species, generated by high resolution melting (HRM) analysis

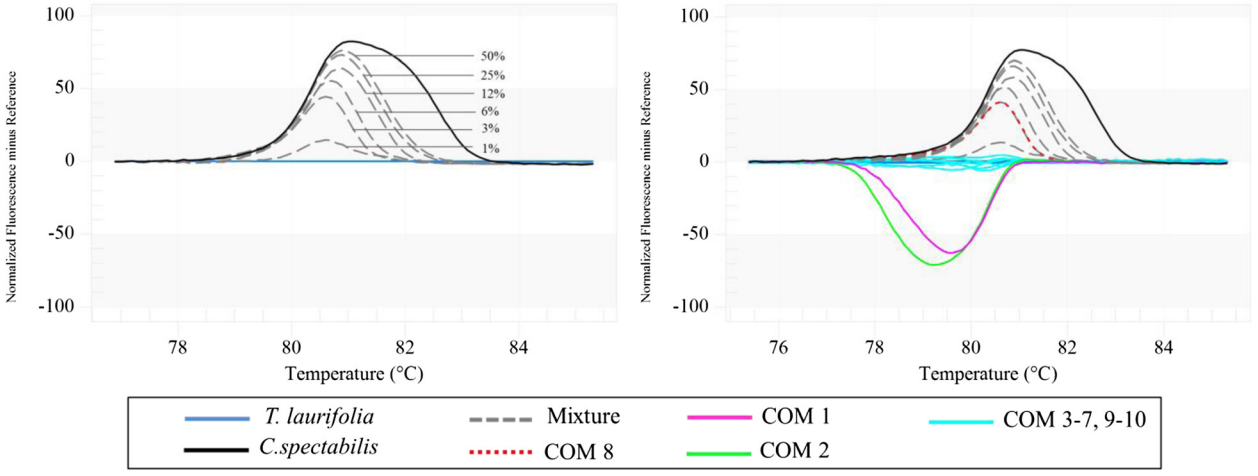

Fig. 2 Melting curves obtained by high resolution melting analysis of the two 'Rang Chuet'species. **a** Specific amplicons and applied to reference mixtures containing 50, 25, 12, 6, 3 and 1 % of *C. spectabilis* in *T. laurifolia*. **b** Difference graph of ten commercial herbal products using *T. laurifolia* as reference species. Data are from a single experiment

sequences to *Moringa oleifera* and *Andrographis paniculata*, respectively (Additional file 1: Table S1). The finding provides evidence that substitution in herbal products sold Thai local market is presented and this substitution could be a serious issue for consumers. Due to the fact that Bar-HRM has allowed us easily determine herbal species in processed products sold on the markets within 2 h. Bar-HRM method developed in this study therefore pose a potential to be a great tool in detection of adulteration and/or substitution in herbal products especially in processed forms.

Conclusions

Several studies have shown that substitution of plant species occurs in herbal medicines, and this in turn poses a challenge to herbal authentication as adverse reactions might be due to substituted ingredients. Bar-HRM has proven to be a cost-effective and reliable method for the identification of species in this study of Thai medicinal plants. The hybrid method of DNA barcoding and High Resolution Melting is dependable, fast, and sensitive enough to distinguish between species. In this study, the tested products were traded as processed powder, which impedes conventional identification. Because of this processing it is almost impossible to identify which herbal species are present in products using morphological characters. The DNA extracted from all products tested yielded a specific amplification product with the designed *rpoC* Bar-HRM primers. The normalized HRM curves for the amplicons, from the two 'Rang Chuet' species (*T. laurifolia* and the toxic *C. spectabilis*) and ten herbal products, based on HRM analysis with barcode marker *rpoC* were easily distinguished, and seven of the ten tested samples were successfully assigned to the *T. laurifolia* species. However, three products were found to contain other plant species or admixture of the two 'Rang Chuet' species. Therefore, the developed method could be easily used for rapid and low-cost authentication of herbal products. Interestingly, designing primer for HRM analysis commonly depends on information in database but here even none of *rpo*C sequences of *Thunbergia* could be found on the database, the *rpo*C primer pair derived from DNA data of other random medicinal plant species is found to be work well in this analysis. It is demonstrated that this method has not only shown the great beneficial value for universality test which might be useful in other medicinal species but also flexibility using DNA region with limited data like *rpo*C.

Additional file

Additional file 1: Table S1. Blast results of two commercial product *rbcL* sequences.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MO planned the study; SS and MO collecting the plant materials, performed the study and conducted the data analysis; MO prepared data for the additional supporting file; SS and MO wrote the manuscript; MO thoroughly revised the manuscript; Both authors discussed the results and approved the final manuscript.

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References

1. Kunle OF, Egharevba HO, Ahmadu PO. Standardization of herbal medicines - A review. IJBC. 2012;4(3):101–12.
2. Oreagba IA, Oshikoya KA, Amachree M. Herbal medicine use among urban residents in Lagos, Nigeria. BMC Complement Altern Med. 2011;11:117.
3. Chan EWC, Lim YY. Antioxidant activity of *Thunbergia laurifolia* tea. JTFS. 2006;18:130–6.
4. Suwanchaikasem P, Chaichantipyuth C, Amnuoypol S, Sukrong S. Random amplified polymorphic DNA analysis of *Thunbergia laurifolia* Lindl. and its related species. JMPR. 2012;6:2955–61.
5. Suwanchaikasem P, Phadungcharoen T, Sukrong S. Authentication of the Thai medicinal plants sharing the same common name 'Rang Chuet': *Thunber gia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* aff. *amada* by combined techniques of TLC, PCR-RFLP fingerprints, and antioxidant activities. ScienceAsia. 2013;39:124–33.
6. Chan EWC, Eng SY, Tan YP, Wong ZC. Phytochemistry and pharmacological properties of *Thunbergia laurifolia*: A Review. Phcogj. 2011;3:1–6.
7. Flores AS, de Azevedo Tozzi AMG, Trigo JR. Pyrrolizidine alkaloid profiles in *Crotalaria* species from Brazil: Chemotaxonomic significance. Biochem Syst Ecol. 2009;37:459–69.
8. Leverett LD, Woods M. The Genus *Crotalaria* (Fabaceae) in Alabama. Castanea. 2012;77:364–74.
9. Schultze AE, Roth RA. Chronic pulmonary hypertension-the monocrotaline model and involvement of the hemostatic system. J Toxicol Environ Health B Crit Rev. 1998;1:271–346.
10. Wonkchalee O, Boonmars T, Aromdee C, Laummaunwai C, Khunkitti W, Vaeteewoottacharn K, Sriraj P, Aukkanimart R, Loilome W, Chamgramol Y, Pairojkul C, Wu Z, Juasook A, Sudsarn P. Anti-inflammatory, antioxidant and hepato protective effects of *Thunbergia laurifolia* Linn. on experimental opisthor chiasis. Parasitol Res. 2012;111:353–9.
11. Prenner GA, Bush A, Wise R, Kim W, Dommier L, Kasha K, Laroche A, Scoles G, Molnar SJ, Fedak G. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. Genome Res. 1993;2:341–5.
12. Yang X, Quiros C. Identification and classification of celery cultivars with RAPD markers. Theor Appl Genet. 1993;86:205–12.
13. Qiu JJ, Li YP. Random amplified polymorphic DNA analysis of eel genome. Cell Res. 1999;9:217–23.
14. Carvalho VP, Ruas CF, Ferreira JM, Moreira RMP, Ruas PM. Genetic diversity among maize (*Zea mays* L.) landraces assessed by RAPD Markers. Genet Mol Biol. 2004;27:228–36.
15. Techen N, Parveen I, Pan Z, Khan IA. DNA barcoding of medicinal plant material for identification. Current Opinion In Biotech. 2014;25:103–10.
16. von Crautlein M, Korpelainen H, Pietilainen M, Rikkinen J. DNA barcoding: a tool for improved taxon identification and detection of species diversity. Biodivers Conserv. 2011;20:373–89.
17. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. PNAS. 2005;102(23):8369–74.
18. Ganopoulos I, Madesis P, Darzentas N, Argiriou A, Tsaftaris A. Barcode High Resolution Melting (Bar-HRM) analysis for detection and quantification of PDO "Fava Santorinis" (*Lathyrus clymenum*) adulterants. Food Chem. 2012;133:505–12.
19. Jaakola L, Suokas M, Haggman H. Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. Food Chem. 2010;123:494–500.
20. Sakaridis I, Ganopoulos I, Argiriou A, Tsaftaris A. A fast and accurate method for controlling the correct labeling of products containing buffalo meat using High Resolution Melting (HRM) analysis. Meat Sci. 2013;94:84–8.
21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013;30:2725–9.
22. Kool M, Korshunov A, Remke M, Jones DTW, Schlanstein M, Northcott PA, Cho YJ, Koster J, Schouten-van Meeteren A, van Vuurden D, Clifford SC, Pietsch T, von Bueren AO, Rutkowski S, McCabe M, Collins VP, Backlund ML, Haberler C, Bourdeaut F, Delattre O, Doz F, Ellison DW, Gilbertson RJ, Pomeroy SL, Taylor MD, Lichter P, Pfister SM. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. Acta Neuropathol. 2012;123:473–84.
23. Bosmali I, Ganopoulos I, Madesis P, Tsaftaris A. Microsatellite and DNA-barcode regions typing combined with high resolution melting (HRM) analysis for food forensic uses: a case study on lentils (*Lens culinaris*). Food Res Int. 2012;46:141–7.
24. CBOL Plant Working Group. A DNA barcode for land plants. Proc Natl Acad Sci. 2009;106:12794–7.
25. Hollingsworth PM. Refining the DNA barcode for land plants. Proc Natl Acad Sci. 2011;108:19451–2.
26. Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S. DNA barcoding detects contamination and substitution in North American herbal products. BMC Med. 2013;11:222.